ROLE OF THE INTERFERON GAMMA/INTERFERON GAMMA RECEPTOR COMPLEX IN SIGNAL TRANSDUCTION

BY

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Interferon gamma (IFNγ) is a multipotent cytokine known for its numerous immunomodulatory activities, including antiviral and antitumoricidal effects. In collaboration with others, I have examined the possible importance of the IFNγ/IFNγ receptor (IFNGR) complex in receptor/cytokine signaling. Expanding on previous peptide studies that identified an IFNGR alpha chain (IFNGRα) cytoplasmic binding site for IFNγ, we have shown that HuIFNγ interacts with both the cytoplasmic domain of human (Hu) IFNGRα chain and soluble murine (Mu) IFNGRα. Through competition studies we have shown that amino acids 95-133 of HuIFNγ and 95-134 of MuIFNγ interact in a species nonspecific manner with the cytoplasmic domains of both human and murine IFNγ receptors. This binding was

inhibited by preincubation of ^{125}I labeled HuIFNγ with peptides corresponding to MuIFNGRα (253-287) showing that this is in fact the region of interaction.

Additionally we have shown the nuclear translocation of the HuIFNGRα, but not the beta (HuIFNGRβ) chain. This nuclear translocation possessed kinetics similar to those of the nuclear translocation of STAT1α, a protein known to undergo nuclear translocation after activation via IFNγ, but itself lacks a nuclear localization sequence (NLS).

We reexamined the importance of the C-terminal sequence of IFNγ. We showed that a truncated HuIFNγ (1-123), although capable of binding to the IFNγ receptor with an affinity similar to that of intact gamma, has less than 1% of the biological activity of intact IFNγ as determined by antiviral assay. HuIFNγ (1–123) lacked the ability to facilitate STAT1α nuclear translocation as efficiently as intact HuIFNγ and had reduced ability to bind to importin α. In addition, we showed that this region of human IFNγ, HuIFNγ 122-132 contains a functional NLS sequence. These findings directly support a hypothesis stating that the NLS of IFNγ mediates the nuclear translocation of STAT1α through their common interaction with the IFNGRα.

CHAPTER 1

INTRODUCTION

Discovery of Interferons

In 1957 Isaacs and Lindermann discovered that the treatment of chick chorio-allantoic membrane fragments with inactivated influenza viruses "interfered" with the ability of fresh influenza virus to replicate within these samples (Isaacs and Lindenmann, 1957). This research led to the identification of a soluble protein (produced in response to viral challenge) which has been termed interferon (IFN). This factor was found to be a member of a large family of proteins characterized by their induction of biological responses and immunological reactivity. Collectively known as interferons, original nomenclature identified these proteins based on their antibody reactivity and the cellular source where they were produced. Currently, IFN nomenclature is based on an agreed convention whereby IFNs are named according to Greek letters. IFNs are currently divided into two main types, type I and type II. Included among type I Interferons are interferons alpha (α) and omega (ω) (produced by leukocytes), beta (β) (produced by fibroblasts), and tau (τ) (produced by trophoblast cells of the conceptus). Currently, the only known type II IFN is gamma (γ), produced by natural killer cells and T cells. Presented in Table 1 is an overview of interferons.

Table I. An overview of interferons

	Cellular source	Biological effects
Type I		
α and ω	leukocytes	antiviral, antiproliferative
β	fibroblasts[a]	antiviral, antiproliferative
τ	trophoblasts	antiviral, antiproliferative Pregnancy recognition
Type II		
γ	lymphocytes	antiviral, antiproliferative

[a] Also produced by macrophages, virally infected cells and epithelial cells (Reviewed in Baron et al. 1991)

Discovery of IFNγ

Wheelock first described IFNγ as an IFN-like inhibitor of the cytopathic effects of the Syndbis virus (1965). IFNγ was first induced in cultures of human leukocytes by phytohemagglutinin (PHA) (Wheelock, 1965). The sole type II interferon, IFNγ, differs from the interferon described by Issacs and Linderman by its instability at pH =2 and its cellular source (generally leukocytes as opposed to non-immune tissues). Electrophoretic mobility studies of IFN derived from leukocytes revealed two distinct peaks of IFN activity of different molecular masses (Stewart and Desmyter, 1975). It was also demonstrated numerous times that antisera raised against "well defined" type I interferons was unable to neutralize the activity of this interferon derived from leukocytes (Younger and Salvin, 1973; Harvell et al., 1975; Valle et al., 1975).

Biological Activity of IFNγ

Interferon gamma possesses antiviral activities similar to that of type I interferons (α,β,ω,τ), however the antiviral activity is substantially lower than that of the interferons α and β (Stuart and Desmyter, 1975). In addition, IFNγ has been shown to act as a potent immunomodulatory molecule. IFNγ has been shown to upregulate the surface expression of the major histocomapatibility complex (MHC) class I and class II antigens on numerous cell types (Baron et al., 1991, Sen and Lengyel, 1992). MHC class I molecules are necessary for the general function of cytotoxic CD 8+ T cells, whereas MHC class II molecules help to present foreign

particles to CD4+ helper T cells. Expression of surface Fc receptors on macrophages (Friedman et al., 1980; Itoh et al, 1980; Weyenbergh et al., 1998) and expression of interleukin 2 receptors on lymphocytes can both be increased by IFNγ (Johnson and Farrar, 1983).

The expression of cell surface receptors is not the only immunomodulatory activity of IFNγ. IFNγ plays a crucial role in the induction of macrophages to a tumoridicidal state (Kleinschmidt and Shultz, 1982; Pace, et al., 1983) and the enhancement of natural killer cell activity. IFNγ plays an important role in antibody (Ab) production. IFNγ acts to suppress Ab production before B cell clonal expansion (Gisler et al., 1974; Sonnenfeld et al., 1977. Activated B cells react to IFNγ by differentiating terminally and by secreting antibodies (Sidman et al., 1984; Leibson et al., 1984).

In addition to the above attributes, IFNγ also has the ability to combat both the formation of tumors and the replication of various intracellular pathogens. IFNγ is capable of inducing numerous genes, including nitric oxide synthase and indoleamine 2,3 dioxygenase which has been shown to mediate IFNγ activity (Struehr et al., 1989; Lyons et al., 1992; Xie et al., 1992). It has also been demonstrated that IFNγ has the ability to inhibit cellular proliferation as relating to tumors (Lee et al., 2000; Kominsky et al., 1998; Hobeika et al., 1998; Knupfer et al., 2000). Some of the biological activities of IFNγ are outlined in Table II.

Table II. General biological activities of IFNγ

Antiviral
Antitumoricidal
Antiproliferative
B cell antibody regulation
Induction of tumor necrosis factor in macrophages
Regulation of natural killer cell activity
Regulation of nitric oxide synthetase production
Induction of indolamine 2,3 dioxygenase
Upregulation of MHC class I and Class II surface expression
Upregulation of cell surface receptors

Characterization and Cloning of IFNγ

IFNγ is a glycoprotein produced by natural killer cells and T cells (both CD4+ and CD8+) of approximately 17 kilodaltons (kd). IFNγ expression is closely linked to the process of T cell activation. In order to closely examine the properties of IFNγ, work was conducted to obtain a purified protein and/or to clone its genes. In 1979 it was discovered that IFNγ would adhere to pore glass beads (Laybord et al., 1979). Utilizing this discovery, a purification scheme was established whereby IFNγ was first adsorbed to glass beads, bound to Concanavalin-A sepharose, gel filtered, and finally HPLC purified (Rinderknecht et al., 1984).

In the early 1980s the cDNA for murine (Gray et al., 1983) and human IFNγ (Gray et al., 1982) was first cloned and expressed. There is a single copy of genes for IFNγ for mice and humans (chromosomes 10 and 21 respectively). The cleavage of a 23 amino acid signal peptide (Devos et al., 1982; Goeddel et al., 1980; Gray et al., 1982b) yields a 143 residue mature protein (purification of natural human IFNγ has revealed some C terminal heterogeneity among IFNγ molecules probably due to end peptidases). Mature murine IFNγ protein consists of 133 amino acids and is similar to human IFNγ in that it has two potential sites for N-linked glycosylation (Gray et al., 1983). Glycosylation does not, however, appear to be a prerequisite for interferon function in that recombinant IFNγ produced in *E coli* is not glycosylated and retains functionality. In nature, IFNγ exists as an anti-parallel dimer where the N-terminus of one molecule interacts with the C-teminus of another (Ealick et al., 1991).

Interferons as Therapy for Human Disease

The use of IFNγ and other IFNs as treatment for disease has shown promise in recent years. Although somewhat controversial, clinical trials using IFNs in the treatment of various diseases has often found success. Currently, the recommended treatment for chronic hepatitis C virus (HCV) infection is three million international units (mIU) of IFNα three times a week for one year (Ahmed and Keeffe, 1999). Clinical trials are currently underway to determine the efficacy of using IFNα as a treatment for multiple sclerosis, in particular the exacerbation-remission form (Cabrera-Gomez and Lopez-Saura, 1999), in addition to IFNβ (Giovannoni and Miller, 1999; Blumhardt, 1999; Arnason, 1999). Further, It has been shown that IFNα is one of the most effective treatments for chronic myeloid leukemia (CML)(Silver et al, 1999) and is still under clinical trials for the treatment of non-Hodgkin's lymphoma (Haas-Statz and Smalley, 1999).

It has been shown that IFNγ is an effective inducer of CD20 on malignant plasma cells in multiple myeloma (MM) at physiological doses (Treon et al, 2000). Studies are currently underway to ascertain the use of this marker for antibody mediated immunotherapy as a possible treatment for MM. It has recently been shown that IFNγ has the ability to modulate the activity of 5-fluorouracil (5-FU), making it more effective in the treatment of colorectal cancer (Makower and Wadler, 1999). IFNγ has also been approved for treatment of chronic granulomatus

disease (CGD), a rare inherited immunodeficiency disease (Meischl and Roos, 1998) and is the recommended treatment in the prevention of disease relapse (Ahlin et al., 1997; Kume and Dinauer, 2000). IFNγ is thought to help restore the ability of phagocytes to produce sufficient amounts of reactive oxygen intermediates to combat this chronic infection (Meischl and Roos, 1998; Weening et al., 1996) and increase the level of nitric oxide production in polymorphonuclear neutrophils (Ahlin et al., 1999). It has been shown that IFNγ is an effective treatment for leishmaniasis (Murray and Delph-Etienne, 2000), a condition whereby ulcers are caused by a parasite of the genus *Leishmania*. It has been suggested that adjuvant-containing antigens that stimulate IFNγ may serve as an effective vaccine against leishmaniasis (Aebischer et al., 2000). IFNγ with and without other anti-parasitic compounds has been shown to control/cure the syndrome through the action of nitric oxide production that kills the intracellular parasite in infected macrophages. It was found that IFNγ has the ability to reduce intracellular bacterial load in murine macrophages, a result that could possibly have future therapeutic applications (Mahon and Mills, 1999). In addition, IFNγ has been shown to enhance the resistance of macrophages to *Chlamydia pneumoniae* infection (Airenne et al., 2000).

Receptor-Mediated Endocytosis

Receptor mediated endocytosis (RME) is one process by which extracellular proteins are internalized by cells. In RME proteins first bind to the extracellular

domain of their specific receptor. Ligand binding then generally induces dimerization and activation of receptor subunits usually by phosphorylation (Vieira et al., 1996). The binding of the ligand to its respective receptor then induces a conformational change that facilitates the binding of clathrin to the cytoplasmic face of the cell membrane surrounding the receptor (Goldstein et al., 1985). Dynamin, a GTPase recruited to the area of clathrin concentration, is thought to induce the invagination of the plasma membrane. Although GTP is required for dynamin action, GTP hydrolysis is not required for the production of this invagination (Schmid and Damke, 1995). Both GTP and ATP, however are required for the budding of this vesicle into the cell. This internalized vesicle then fuses with other vesicles (including lysosomal vesicles) and travels to their desired area of the cell. It is then thought that the ligand is degraded and that the receptor is likewise degraded and/or recycled (Goldstein et al., 1985).

Functional Sites on IFNγ Molecule

Monoclonal antibody studies have indicated that both the amino terminal and carboxy terminal epitopes of IFNγ are important for function (Schreiber et al., 1985; Russell et al., 1986). Both murine and human IFNγ were inhibited from binding to their respective cell surface receptors through the use of monoclonal antibodies against the N terminal regions of these proteins, inhibiting both their antiviral activity on fibroblasts and their induction of macrophage cytotoxic activities (Johnson et al., 1982; Russell et al., 1986; Farrar and Schreiber, 1993).

The importance of the first 39 N-terminal amino acids of both human and murine IFNγ was displayed when synthetic peptides constructed corresponding to these regions were capable of inhibiting IFNγ binding to receptor proteins (Magazine et al., 1988). It was also shown that the removal of the first 9 amino acids of murine IFNγ resulted in a protein without any observable antiviral activity (Zavodny et al., 1988).

The importance of the C terminus has also been examined. Although there is no significant homology between IFNγ proteins of different species, a strictly conserved region of 4-5 basic amino acids at the C-terminal region is observed between species (Ealick et al., 1991). It has been shown that monoclonal antibodies directed against this region of murine IFNγ resulted in the abrogation of both the antiviral and macrophage priming activities of IFNγ (Schreiber et al., 1985). In the same respect, monoclonal antibodies against the basic residues or the substitution of these amino acids with non basic amino acids resulted in a severely diminished induction of biological response (Arakawa et al., 1986; Leinikki et al., 1987; Wetzel et al., 1990; Lundell et al., 1991). Interestingly it was shown using synthetic peptides that these C-terminal amino acids are involved in receptor binding in a region that is different from the region where the amino terminal end binds (Griggs et al., 1992).

The Interferon Gamma Receptor and Signal Transduction

The type II interferon (IFN) IFNγ is a multipotent cytokine secreted by activated T cells and natural killer cells that is responsible for the modulation of many facets of the immune response. IFNγ exerts its effects on the surface of target cells through interaction with a specific receptor complex. The IFNγ receptor complex (IFNGR) is a heterodimeric complex consisting of an α subunit, IFNGRα (90 kDa), and a β subunit (60 kDa), IFNGRβ (Bach et al., 1997; Pestka et al., 1997). The IFNGRα subunit binds IFNγ with high affinity, whereas the IFNGRβ, although contributing to ligand binding and required for signaling (Farrar and Schreiber, 1993; Hemmi et al, 1994; Soh et al., 1994), attaches to IFNγ with a significantly lower affinity (Bach et al., 1997; Pestka et al, 1997). The active ligand-receptor complex consists of a dimer of IFNγ bound by two molecules each of IFNGRα and IFNGRβ. After ligand binding, two tyrosine kinases are activated at the cytoplasmic domains of these subunits: JAK1 by constitutive association with IFNGRα (Farrar et al., 1991; Greenlund et al., 1994; Kaplan et al., 1994) and JAK2 by constitutive association with IFNGRβ (Bach et al., 1996). The cytoplasmic domain of the IFNGRα contains additional signaling sites that include a membrane proximal dileucine motif domain required for receptor internalization and a domain for binding the transcription factor STAT1α, tyrosine 440 (Bach et al., 1997; Pestka et al, 1997). This STAT1α binding site is activated on the cytoplasmic domain by phosphorylation mediated by JAK1 and JAK2. Subsequently, STAT1α is tyrosine

phosphorylated and forms a dimer that is translocated to the nucleus by as yet unknown mechanisms. STAT1α is ultimately thought to drive IFNγ-specific gene regulation. In light of the events at the IFNGRα cytoplasmic domain, the role of IFNGRβ appears to be limited to bringing JAK2 in proximity to the IFNGRα to initiate or participate in the signaling events (Bach et al., 1997; Pestka et al., 1997). Figure 1 shows the current model for IFNγ signal transduction.

Previous studies have shown that besides these signaling events leading to translocation of the transcription factor STAT1α to the nucleus, the ligand, IFNγ, when bound to the receptor complex is itself rapidly transported to the nucleus via receptor-mediated endocytosis (Bader et al., 1994; Macdonald et al., 1986). We have recently shown that nuclear import of IFNγ is driven by a specific nuclear localization sequence in the C-terminus of IFNγ (Subramaniam et al., 1998). Further, studies have suggested that internalization of the IFNγ receptor may be linked to signal transduction (Farrar et al., 1991). These observations question the simple assumption that receptor endocytosis is strictly a recycling mechanism for either receptor and/or ligand. Also, given the multimeric nature of the IFNγ receptor complex, the individual fates of the IFNGRα and IFNGRβ subunits are unknown. This question becomes particularly relevant in light of the fact that the only route for intracellular delivery of ligand required for subsequent nuclear transport of the ligand is via the process of receptor-mediated endocytosis.

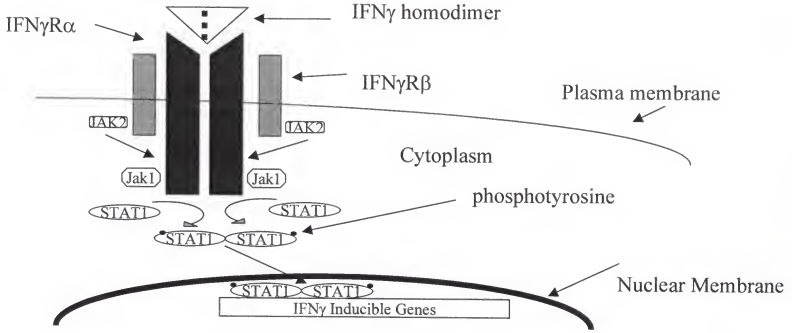


Figure 1. The current model of IFNγ signal transduction. IFNγ binds the IFNGR and induces a cascade of intracellular events. JAK2 transfers from the IFNGRβ to the IFNGRα chain. Both JAK1 and JAK2 are phosphorylated and then phosphorylate tyrosine 440 of the IFNGRα chain. STAT1 then docks on tyrosine 440, becomes phosphorylated, homodimerizes, and translocates to the nucleus where it activates the transcription of gamma inducible genes.

Nuclear Translocation of STAT1α

STAT1α is translocated to the nucleus through the use of the Ran/importin pathway (Sekimoto et al 1996 and Sekimoto et al 1997). In this pathway, proteins destined to travel to the nucleus interact with a heterodimeric protein known as importin which consists of an α and β subunit. Nuclear proteins interact with the α subunit via what is known as a nuclear localization sequence (NLS). Nuclear localization sequences utilized by the Ran/importin pathway generally consist of a cluster of basic amino acids, or two short clusters of basic amino acids separated by a spacer of variable length (Gorlich and Mattaj, 1996). One of the best known simple polybasic NLS sequences is that of the SV40 T-antigen, KKKRK. The β subunit of importin mediates the binding of the NLS bearing protein/importin α complex to Ran GTPase, which is present at the nuclear pore complex. Ran GTPase then mediates the transport of the protein-containing complex through the nuclear pore requiring the hydrolysis of both GTP and ATP to provide the energy necessary for this process to take place (Gorlich and Mattaj, 1996).

This NLS region, however has not been identified on STAT1. Mutational analysis of STAT1 failed to reveal a conventional NLS capable of binding to NPI-1, which is a homologue of Importin α (Sekimoto et al., 1997). The absence of this polybasic NLS in STAT1 gives credence to the hypothesis that another molecule is supplying the necessary NLS, thus assisting in the nuclear transport of STAT1 (Johnson et al., 1998).

Experimental Rationale

Although the associated biological activities of IFNγ occur after it binds to the high-affinity extracellular domain of the receptor (Pestka et al., 1987), at least three independent lines of experimentation have identified an intracellular role for IFNγ in cell activation. These include the observations that 1) HuIFNγ delivered by a liposome vector was able to activate murine macrophages to a tumoricidal state (Fidler et al., 1985), 2) secretion-defective HuIFNγ expressed in murine fibroblasts triggered antiviral activity (Sanceau et al., 1987), and 3) microinjected HuIFNγ-induced Ia expression in murine macrophages (Smith et al., 1990). The activity of HuIFNγ in these murine cells defies the well known species specificity of exogenously applied HuIFNγ which has no activity on murine cells. Thus, the IFNγ molecule most likely interacts with some intracellular element(s) to induce a biological response. Recently, through the use of lipopeptides, additional evidence was produced supporting an intracellular role of interferon gamma. IFNγ C-terminal peptides, which when exogenously applied to cells do not confer detectable biological activity, when conjugated to a lipid moiety (which allows penetration through the cell membrane) were able to upregulate the expression of MHC class II molecules and confer viral protection to the treated cells (Thiam et al., 1998).

Previously, IFNγ binding sites were identified on the cytoplasmic domain of the soluble MuIFNGRα chain subunit using synthetic peptides (Szente et al., 1994;

Szente and Johnson, 1994). Briefly, C-terminal peptides MuIFNγ (95-133) and huIFNγ (95-134) bound to the cytoplasmic domain of the MuIFNGRα chain at residues 253-287. Binding to this membrane proximal region was demonstrated with peptides to the cytoplasmic domain of the receptor as well as with fixed/permeabilized mouse L cells where site-specific antibodies to residues 253-287 specifically blocked binding. The C-terminus of IFNγ contains a polycationic sequence that is required for intracellular binding (Szente et al., 1994; Szente et al., 1996). Functionally, the C-terminal peptide induced an antiviral state and up-regulation of MHC class II molecules when taken up by pinocytosis by a macrophage cell line (Szente et al., 1994). Thus, intracellular binding of IFNγ C-terminus to the receptor cytoplasmic domain is associated with biological activity. Adjacent to this receptor cytoplasmic domain region is a binding site for the tyrosine kinase JAK2 (Igarashi et al., 1994). JAK2 binding to this site is enhanced by IFNγ C-terminus (Szente et al., 1995).

Because the above binding experiments involved only peptides to the MuIFNGR cytoplasmic domain, we have subcloned and expressed the HuIFNGR cytoplasmic domain to evaluate its binding site specificity and relative affinity for HuIFNγ. Through direct and competitive binding experiments, we show that the newly expressed HuIFNGR cytoplasmic domain protein can specifically bind both HuIFNγ and MuIFNγ via their C-terminal regions.

We mentioned previously that IFNγ has been shown to rapidly translocate to the nucleus through indirect means (Bader and Wietzerbin, 1994; MacDonald et al., 1986). Two putative NLS sequences have been identified previously within HuIFNγ

(Bader and Weitzerbin, 1994) and a functional polycationic NLS sequence has been identified within MuIFNγ (Subramaniam et al., 1998). It has therefore been established that the carboxy region of IFNγ plays a significant role in IFNγ signal transduction, which is probably not due to interaction with the extracellular domain of IFNGR. In addition to the intracellular roles of IFNγ mentioned earlier, and the interaction of the C-terminus of IFNγ with the cytoplasmic domain of the IFNGRα chain, crystallographic data showing the interaction of IFNγ with the extracellular domain of the IFNGRα failed to show any interaction between the C-terminus of IFNγ and the extracellular domain of the receptor (Walter et al., 1995). It therefore seems likely that a major portion of this signal transduction modulation occurs after the cellular internalization of IFNγ. For this reason we have studied those cellular events that surround cellular activation via IFNγ.

Although IFNγ nuclear translocation has been shown previously, the actuality and functionality of this event remains controversial. Insulin, like many other Jak/STAT utilizing cytokines has been shown to translocate to the nucleus, (Shah et al., 1995) through the use of direct labeling with nanogold. In order to shed light on the significance of nuclear translocated IFNγ we labeled HuIFNγ with nanogold and observed its nuclear translocation.

IFNγ is translocated to the nucleus, has intracellular biological activity and interacts with the cytoplasmic domain of the IFNγ receptor. Many of IFNγ's biological activities are attributed to the nuclear translocation of STAT1, a protein that binds to tyrosine 440 of the cytoplasmic domain of the IFNγRα chain after cellular stimulation by IFNγ. Although STAT1 is nuclear translocated via the

ran/importin pathway (Sekimoto et al 1996, and Sekimoto et al., 1997) as we have previously noted, it lacks the NLS necessary for nuclear translocation. We therefore propose a chaperone model whereby STAT1 undergoes nuclear translocation via a complex with another NLS containing protein. Because IFNγ contains an NLS and STAT1 is known to interact with the IFNγRα, it is therefore logical that we examine the IFNγRα after IFNγ stimulation. We show that after IFNγ binds to its receptor the IFNGRα subunit of the receptor, like the ligand, is rapidly internalized and translocated to the nucleus. In marked contrast, the IFNGRβ chain does not undergo endocytosis and nuclear translocation.

It has been shown that under certain conditions the C-terminal region of IFNγ is sufficient for biological activity (Szente et al., 1994). We have attempted to answer the question of whether or not IFNγ posseses biological activity in the absence of this region. We have expressed a HuIFNγ devoid of the polycationic C-terminal region and discovered that although capable of binding to the extracellular domain of the receptor, it has both severely reduced antiviral activity and ability to nuclear translocate STAT1. Finally, we have tested to see if HuIFNγ contains a functional NLS and found that this was indeed the case.

CHAPTER 2
MATERIALS AND METHODS

Materials

Recombinant human IFNγ (HuIFNγ) was purchased from BioSource International. The antibodies used in these experiments were as follows: rabbit polyclonal IgG raised against a peptide corresponding to amino acids 466-485 of the HuIFNGRα subunit (Santa Cruz Biotechnology), rabbit polyclonal IgG raised against a peptide corresponding to amino acids 318-337 of the HuIFNGRβ chain (Santa Cruz Biotechnology), goat polyclonal IgG raised against a peptide corresponding to amino acids 702-739 of human STAT1α (R & D Systems). Polyclonal phospho-STAT1 (Tyr701) antibody was purchased from New England BioLabs. $Na^{125}I$ was purchased from Amersham. B-PER™ Bacterial Protein Extraction Reagent for purification of IFNγ (1-123) was from Pierce (Rockford, IL). Fluorescein-5-isothiocyanate was obtained from Molecular Probes.

Preparation of Nanogold/ IFNγ Complex

Interferon gamma (0.02 mg) was added to sufficient SHS nanogold conjugate (Nanoprobes Inc.) to label 1.2 nMoles of amine sites. The reaction mixture was placed at 4°C overnight. The reaction was quenched by adding 5 µl of 1M tris-HCl. Uncoupled HuIFNγ was separated from the IFNγ/nanogold complex via gel filtration using sephadex 75 (Amersham).

Nuclear Translocation of IFNγ/Nanogold Conjugate

Human WISH cells were plated at $2x10^6$ cells on a 60x15 mm tissue culture dish and allowed to adhere overnight. The culture dishes were allowed to cool for 30 minutes at 4°C and then incubated with 10 ml of 12.25 nM HuIFNγ/nanogold conjugate or BSA/nanogold conjugate in the cold room. The tissue dishes were then transferred to a 37°C incubator for 15 minutes. The treated cells were then rinsed 3x with ice cold EMEM + 10% FBS followed by rinsing with 0.1 M cacodylate buffer (3x) to remove unbound protein. Partial fixation of samples was accomplished by incubation of samples in 0.2 M cacodylate buffer containing 1% paraformaldehyde and 0.5% tween for 15 minutes at 4°C. The cells followed by washing 3x with nanopure water. Gold particles conjugated to IFNγ within cells were then silver intensified for 6 minutes according to manufacturer's instructions (Nanoprobes, Inc.) for visualization via electron microscopy followed by vigorous washing with nanopure water (5x). Fixation was then completed by subjecting samples to 0.2 M cacodylate buffer containing 2% glutaraldehyde and 2% paraformaldehyde for 2 hours at 4°C. The cells were then dehydrated through a series of graded ethanols followed by acetone. The samples were then embedded in Spur's plastic and polymerized at 60°C. The plastic was then ultrathin sectioned on an ultramicrotome and observed via transmission electron microscopy using a Zeiss EM 10CA microscope.

Radioiodinations

Radioiodinations were performed as previously described (Szente et al., 1994). HuIFNγ (5 μg) was radioiodinated by combining 10 μl with 5 μl (500 μCi) $Na^{125}I$ (16.9 mCi/μg, Amersham) in the presence of 25 μl of 0.15 M potassium buffer, pH 7.4, and 10 μl Chloramine-T (5 mg/ml) for 2 minutes. After neutralization of the reaction with 10 μl of sodium metabisulfite (10 mg/ml), potassium iodide (70 mg/ml), and BSA (20 mg/ml), the reaction mixture was chromatographed over a 10-ml Sephadex G-10 column equilibrated with a Tris/NaCl/BSA buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.33 mg/ml BSA). Fractions of 500 to 600 μl were collected, and the fraction containing the greatest activity was used for receptor binding studies. The specific activity of ^{125}I-IFNγ was 90-120 μCi/μg of protein.

Binding Assays

Binding assays were performed as previously described (Szente et al., 1994). EIA/RIA plates (Costar, Cambridge, MA) were seeded with HuIFNγR cytoplasmic domain protein or soluble MuIFNGR (10 ng) or MuIFNGR peptide (253-287) in 0.1 M carbonate/bicarbonate buffer, pH 9.6, and allowed to incubate for 18 h at 4°C. The remainder of the assay was performed at room temperature. Plates were washed three times with wash buffer (0.15 M NaCl, 0.05% Tween) then blocked for 1 h in 5% Carnation powdered milk dissolved in PBS. Following this, ^{125}I-HuIFNγ was added to the wells at a final concentration of 5 nM for 1.5 h. For competition assays, competitors were incubated in the wells for 1 h prior to the addition of ^{125}I-HuIFNγ. In the case of saturation assays, various concentrations of

HuIFNγ were added to the wells for 1 h prior to the addition of ^{125}I-HuIFNγ (10 nM). When HuIFNGR cytoplasmic domain protein or soluble MuIFNGR were used as competitors, ^{125}I-HuIFNγ was preincubated with the competing receptor in a microcentrifuge tube for 1 h and then added to the plate wells for 1.5 h. The wells were then removed and the radioactivity quantified in a scintillation counter (Beckman instruments, Irvine, CA). Saturation and competition data were analyzed with the Equilibrium Binding Data Analysis (EBDA) program for "cold" saturation binding and ligand displacement analysis (McPherson, 1983; McPherson and Summers, 1983; McPherson, 1985; McPherson, 1985).

Binding of ^{125}I-IFNγ to WISH cells

The binding of ^{125}I-IFNγ to confluent WISH cells was performed at 4°C using a standard "cold saturation" procedure as previously described for protein binding studies above. Data were analyzed using the LIGAND computer program for determination of binding constants (McPherson, 1985). The Scatchard curve in Figure 8 was replotted from values from the program output.

Immunofluoresence

WISH cells were grown on tissue culture treated slides (Falcon) at 3 x 10^5 cells per slide. Just before use, the cells were washed with ice-cold culture medium and brought to 4°C. Cells were then incubated at 4°C with IFNγ (20,000 units/ml) in ice-cold culture medium for 1.5 hr. Cellular events were initiated by transferring cells to 37°C and incubation for the indicated periods of time. After the appropriate

times, the cells were immediately fixed in methanol (-20°C), and then permeabilized using 0.5% Triton X in 100 mM Tris-HCl, 0.9% NaCl (TBS) for 10 minutes. Slides were washed in TBS and non-specific sites were blocked with TBS containing 5% dried nonfat milk containing 0.1% Triton X, and the cells incubated in the same blocking solution with antibodies against IFNGRα, IFNGRβ or STAT1α, either alone or in combination as indicated. After 1.5 hours of incubation at room temperature cells were washed with TBS containing 0.1% Triton-X, followed by incubation with FITC conjugated donkey anti-goat IgG and/or Texas red conjugated donkey anti-rabbit IgG, as the case may be. The slides were again washed repeatedly and stained with a solution of DAPI according to manufacturer's recommendations (Molecular Probes). Following washing, cells were mounted in Prolong antifade solution (Molecular Probes), covered with a coverslip, and sealed with nail varnish.

The images observed were obtained through the use of an Olympus 1X70 deconvolution microscope under oil immersion 60x objective and an auxiliary 1.5x magnification. Subsequent to the acquisition of these images, they were then further deconvolved through the use of Applied Precision's Delta Vision deconvolution algorithm [reviewed in Hiraoka et al., 1991].

In the case of experiments using double-staining for the IFNGRα chain and the IFNGRβ chain (Figure 11) within the same cells, the cells were first stained for IFNGRβ. Following washing of the secondary antibody, the cells were incubated with FITC-conjugated IFNGRα chain antibodies for 1 hour. All other procedures are identical to that of the other immunofluorescence experiments.

Quantitation of fluorescence was performed on images using the NIH Image software. The mean fluorescence (f) intensity from approximately equal areas in the cytoplasm (fc) and the nucleus (fn) from each cell within a field was measured. The areas were chosen arbitrarily within cells and across fields. This was designed to give truly average values. The ratio fn/fc for each cell from treated samples was subtracted against the average fn/fc ratio from measurements on untreated cells, and the resulting values, Fn/Fc, were averaged for each field. Averaged Fn/Fc ratios were then plotted against time or dose of IFN treatment.

Conjugation of IFNGRα Chain Antibodies with FITC:

For IFNGRα antibody labeling, purified rabbit anti-human IFNGRα chain antibodies were obtained from Santa Cruz Biotechnology. Fluorescein-5-isothiocyanate was obtained from Molecular Probes. Ten mgs FITC was dissolved in anhydrous DMSO. The FITC solution was then added to purified IFNGRα chain antibodies at a concentration of 80 μg per mg of antibody. This mixture was then incubated at room temperature for 1 hour. The unreacted FITC was removed via gel filtration.

Preparation of Cytosolic and Nuclear Extracts

IFNγ (20,000 units/ml) in ice-cold culture medium was added to previously cooled (4°C) flasks containing confluent WISH cells, and the cells incubated for 1 ½ hours at 4°C. For initiation of cellular events, the flasks were incubated at 37°C for the indicated time periods. After these time periods, the cells were removed by

scraping, collected by centrifugation at 4°C for 4 min, and cell pellets were immediately flash-frozen in liquid nitrogen. The cells were lysed in buffer containing 10 mM Hepes (pH 7.9), 40 mM KCl, 50 mM NaF, 3 mM $MgCl_2$, 20 mM β-glyceroylphosphate, 1 mM DTT, 5% glycerol, 0.2% NP-40, 1 mM PMSF, 2 mM sodium orthovanadate, and 1 μg/ml each of pepstatin, aprotinin, and leupeptin for 10 minutes on ice. Samples were centrifuged for 10 minutes at 1,000 rpm at 4°C, and supernatants containing the cellular cytosolic fraction were then collected and saved for further experimentation (Greenburg and Bender, 1994).

The pellets containing isolated nuclei were carefully resuspended in lysis buffer and washed twice with lysis buffer. The presence of isolated nuclei was verified by trypan blue staining, and nuclei counted. Nuclear extracts were prepared from the intact nuclei by lysis, for 30 minutes on ice, using a high salt buffer consisting of 20 mM Hepes (pH 7.9), 420 mM KCl, 1.5 mM $MgCl_2$, 50 mM NaF, 0.2mM EDTA, 0.5 mM DTT, 2 mM sodium orthovanadate, 25% glycerol, 0.5 mM PMSF, 1μg/ml each of pepstatin, aprotinin, and leupeptin, and centrifugation for 15 minutes at 14,000 rpm at 4^0C to remove the insoluble fraction. The nuclear extracts were assessed for contamination with cytosolic proteins by measuring the activity of the cytosolic enzyme lactate dehydrogenase. Nuclear extracts contained less than 1% of the cytosolic specific activity of this marker enzyme.

Immunoprecipitation Experiments

Equal amounts of protein, within a given experiment, for each of the cytosolic extracts and nuclear extracts were incubated at 4^0C with the indicated

antibodies for 2 hr. Immunoprecipitation and immunoblotting of immune complexes were performed as previously described (Subramaniam and Johnson, 1997).

For co-immunoprecipitations using ^{125}I-labeled IFNγ, human WISH cells (see legends) were incubated at 4°C with 0.33 μg/ml (3300 units/ml) of ^{125}I-labeled human IFNs for 1 hour. Cells were then shifted to 37°C for the appropriate time periods. Control cells were maintained at 4°C. Following incubation at 37°C, cells were washed three times with ice-cold growth medium, once with ice-cold PBS. Control cells were processed last. Cells were dislodged by scraping into cold PBS, and flash-frozen in liquid N_2, for storage at –80°C till use. Cells were lysed at 4°C into lysis buffer (see above), to provide cytoplasmic extracts.

For immunoprecipitation, equal protein amounts of lysates were immunoprecipitated using STAT1α antibodies. Immunoprecipitates were washed once with 50 mM Tris-HCl, pH 6.8, containing 2 mM sodium orthovanadate, and immune complexes separated on SDS-PAGE. Following transfer of proteins to nitrocellulose, ^{125}I-IFNγ was detected by autoradiography. Immunoprecipitated STAT1α was detected by immunoblotting with anti-STAT1α antibodies.

Purification of Recombinant Human IFNγ (1-123):

The expression vector for the C-terminal truncated mutant of human IFNγ, IFNγ (1-123), was kindly provided by Dr. Mark R. Walter (University of Alabama at Birmingham, Birmingham, AL). The protein was expressed in *E. coli* grown at 37°C on a shaker in LB medium (1% bacto-tryptone, 0.5% Bacto-yeast, and 1%

NaCl) supplemented with 100 mg/ml ampicillin (Green et al, 1989), and IFNγ (1-123) and was purified from inclusion bodies (Haelewyn and Ley, 1995). Briefly, protein expression was induced via the addition of isopropylthiogalactoside (IPTG) for 14 hours. Cells were harvested and lysed using B-PER Bacterial protein extraction reagent (Pierce), to which 0.2 mg/ml lysozyme had been added, according to manufacturer's recommendations. The lysate was subjected to bovine pancreatic ribonuclease A and bovine pancreatic deoxyribonuclease I at a final concentration of 1μg/ml for 30 min. at 25°C. Inclusion bodies were recovered by centrifugation at 12,000 g for 30 min. Inclusion bodies were washed extensively with extraction buffer (B-PER reagent), Following centrifugation and washing, the inclusion bodies were solubilized in 6 M guanidine hydrochloride in extraction buffer for 1 hr at 37°C. Insoluble material was removed by centrifugation, and the supernatant containing the protein was dialyzed slowly at 4°C with a decreasing gradient of guanidine hydrochloride until the protein was suspended in 10 mM Tris Buffered Saline containing 0.1 M guanidine hydrochloride. The purity of the renatured protein was determined by a combination of SDS-PAGE analysis and Western blotting with antibodies to the N-terminus of IFNγ. In the assays described here the final concentration of the guanidine hydrochloride did not exceed 0.1 mM, which was not found to affect cell growth or viability.

Cell Culture

Human HeLa cells (ATCC) were grown in DMEM containing Penicillin/Streptomycin and 10% Fetal Bovine Serum. Cells were plated onto coverslips 24 h before use. Human WISH cells (ATCC) were grown in EMEM

containing Penicillin/ Streptomycin and 10% Fetal Bovine Serum. Cells were plated either 24 or 48 hours prior to use as indicated for each experiment.

Peptide Synthesis

Peptides used in this study were synthesized on a PerSerptive Biosystems 9050 automated peptide synthesizer using Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry (as detailed in Szente et al., 1996).

Preparation of Import Substrates (APC conjugates)

Allophycocyanin (APC) activated with the bifunctional cross-linker succinimidyl 4-(N-maeimidomethyl)cyclohexane-1-carboxylate (SMCC) was purchased from Prozyme (San Leadro ,CA) and used according to manufacturer's suggestions. Briefly, peptides reduced with dithiotheritol (50 mM) were coupled at a 1: 1 or 1:2 molar ratio (APC: peptide) in 5 mM MES, pH 6.0, containing 5 mM EDTA. After the initial separation of uncoupled peptides by gel filtration through an Econo 5DG column (Bio Rad) in 20 mM Hepes, pH 7.3, any residual peptide was removed by repeated concentration in the same buffer through a Centricon 50 ultrafiltration unit (MWCO 50,000; Amicon, Inc., Beverly, MA) and the conjugate stored at 4°C. The coupling efficiency (peptide/ APC) and peptide removal were established by SDS-polyacyrlamide gel electrophresis.

Nuclear Import Assays

Transport assays with human Hela cells were based on methods previously described (Adam, 1992). Cells grown on coverslips were washed at 4°C with transport buffer: 20 mM Hepes, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol, 10 μg/ml each of leupeptin, pepstatin, and aprotinin. Cells were permeabilized with digitonin (at 40 μg/ml) in transport buffer for 5 min at 4°C. After washing with transport buffer, cells were incubated with the transport reaction mixture for 30 min at 30°C. Complete reaction mixtures (60μl final volume) contained 20 mM HEPES, pH 7.3; 110 mM potassium acetate; 2 mM magnesium acetate; 1 mM EGTA; 2 mM dithiothreiotol; 10 μg/ml each of leupeptin, pepstatin, and aprotinin; 0.5 mM GTP; 2.5 mM ATP; 5 mM phosphocreatine (Calbiochem); approximately 200 nM appropriate import substrate and 20 μl of rabbit reticulocyte lysate (untreated; Promega, Madison, WI). Coverslips were washed in transport buffer containing 1% bovine serum albumin, mounted on slides, and observed under a fluorescence microscope (cooled-CCD deconvolution microscopy)

For ATP depletion experiments reticulocyte lysates were first treated with a mixture of hexokinase (~300 units/ml), glucose (8mM), and apyrase (0.2 units/ml) at 30°C for 15 min before the addition of the rest of components. For GTP dependence, GTP was omitted from the reaction mixture and the reticulocyte lysate was incubated at room temperature with the analog GTPγS (Calbiochem) at 5 nM before the addition of other components.

For peptide competition experiments, unlabeled peptides were added in excess as described in various experiments, calculated with respect to APC, in the presence of all other components with the exception of the import substrate. After incubation at room temperature for five minutes, the APC substrate was added and the mixture incubated with the cells.

CHAPTER 3
RESULTS

Nuclear Translocation of IFNγ

Various techniques were utilized in order to observe of nuclear translocation of IFNγ. We originally attempted to use colloidal gold labeled IFNγ, however we were unsuccessful. It appears that the size of the gold particle changed the properties of the internalization of the molecule. It is also possible that the colloidal gold/IFNγ conjugate, which is very dependent on pH, dissociated within the lysosomal vesicles leaving the colloidal gold to accumulate in multivesicular bodies while the dissociated protein continued its respective tasks. There is evidence supporting the dissociation of the colloidal gold/protein conjugate within the low pH lysosomal vesicles (Shah et al., 1995). We then turned to the use of nanogold, which is a 1 nm gold particle that has the ability to covalently bind to proteins (Shah et al., 1995). The nanogold particle/IFNγ complex was then internalized as any other protein would be internalized due to the small size of the nanogold particle. Subsequent to the preparation of cell samples as described in Materials and Methods, the internalization and/or nuclear translocation of the nanogold/IFNγ complex was visualized within the treated cells via electron microscopy. It can be observed in Figure 2 that the IFNγ/nanogold complex did in fact undergo nuclear translocation within 15 minutes as illustrated by the dark uniform circles located

Figure 2. Nuclear translocation of HuIFNγ. WISH cells were treated with HuIFNγ complexed to nanogold as described in "Materials and Methods". The treated cells were prepared as indicated within "Materials and Methods" such that it was possible to observe them via electron microscopy. The dark uniform circles denoted by arrows illustrate the location of the interferon molecules.

within the nucleus, indicated by arrows. We also subjected WISH cells to the same treatment using uncoupled nanogold particles as a control. Unbound nanogold particles were not observed within these cells thus ruling out the possibilty that the gold particles observed in the nucleus are the result of nonspecific activity (data not shown). In addition, we conducted similar experiments using nanogold complexed to bovine serum albumin (BSA). Again, no particles were observed within these samples (data not shown).

Interaction of IFNγ with the IFNγR Cytoplasmic Domain

Previously, synthetic peptides representing different regions of HuIFNγ and MuIFNγ were used to identify binding sites on the extracellular and cytoplasmic domains of the soluble MuIFNGR (Szente and Johnson, 1994; Szente et al., 1994). Here, we wanted to determine whether the cytoplasmic domain of the HuIFNGR, expressed without the structural and functional attributes of the extracellular domain, could function to bind IFNγ with the same relative affinity and site specificity determined for the cloned soluble MuIFNGR containing the extracellular and cytoplasmic domains (Szente and Johnson, 1994; Szente et al., 1994).

Initially, we established the ligand specificity of the newly expressed HuIFNGR cytoplasmic domain in solid-phase competitive binding assays (Figure 3). Binding of ^{125}I-HuIFNγ was effectively competed with 800 nM unlabeled

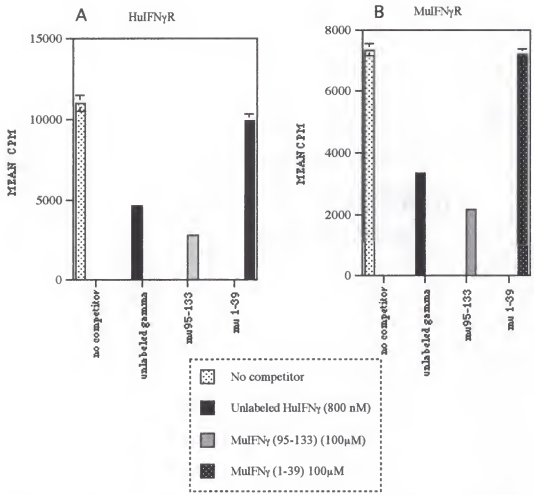


Figure 3. Effect of HuIFNγ and IFNγ peptides on ^{125}I-HuIFNγ binding to HuIFNγR and MuIFNγR. Soluble HuIFNγR cytoplasmic domain protein (10 ng) (Panel A) or MuIFNγR (10 ng) (Panel B) were absorbed to the wells of microtiter plates for 18 h at 4°C. Binding of ^{125}I-HuIFNγ (5 nM) (specific activity of 112 μCi/μg) was competed in each well with unlabeled HuIFNγ (800 nM), C-terminus MuIFNγ(95-133) (100 μM), or N-terminus MuIFN((1-39) (100 μM). Cpm data represent the mean of triplicate wells.

HuIFNγ and 100 μM C-terminus MuIFNγ peptide, IFNγ (95-133). The N-terminal MuIFNγ peptide, MuIFNγ (1-39), previously shown to bind exclusively to the extracellular domain of the receptor (VanVolkernburg et al., 1993), did not compete for binding in these experiments. The data show that binding of HuIFNγ to intracellular regions of the HuIFNGR and MuIFNGR was mediated through cytoplasmic domain binding to the C-terminal region of HuIFNγ.

To further test the specificity of HuIFNγ binding, we determined dose responses of the inhibition of ^{125}I-HuIFNγ binding to the HuIFNGR cytoplasmic domain protein and the cytoplasmic domain of the soluble MuIFNGR by HuIFNγ and C-terminal peptides HuIFNγ (95-134) and MuIFNγ (95-133). The effective concentrations of unlabeled HuIFNγ needed to block half-maximal binding to the HuIFNGR (Figure 4A inset) and MuIFNGR (Figure 4B inset) cytoplasmic domains (EC_{50}) were similar, 1.5×10^{-8} M and 3×10^{-8} M, respectively. The EC_{50} of C-terminus HuIFNγ (95-134) and MuIFNγ (95-133) needed to block binding to the HuIFNGR cytoplasmic domain protein were 7×10^{-6} M and 3.5×10^{-6} M, respectively (Figure 4A). Similar EC_{50} were obtained for peptide inhibition of binding of ^{125}I-HuIFNγ to the MuIFNGR cytoplasmic domain (Figure 4B). Binding control IFNγ peptide, MuIFNγ (1-39), was ineffective at blocking binding. Thus, HuIFNγ was 200 to 500 times more effective than the peptides in blocking binding of ^{125}I-HuIFNγ to the receptor cytoplasmic domain, which suggests that the C-terminus of the intact HuIFNγ was better recognized by the receptor than C-terminus alone.

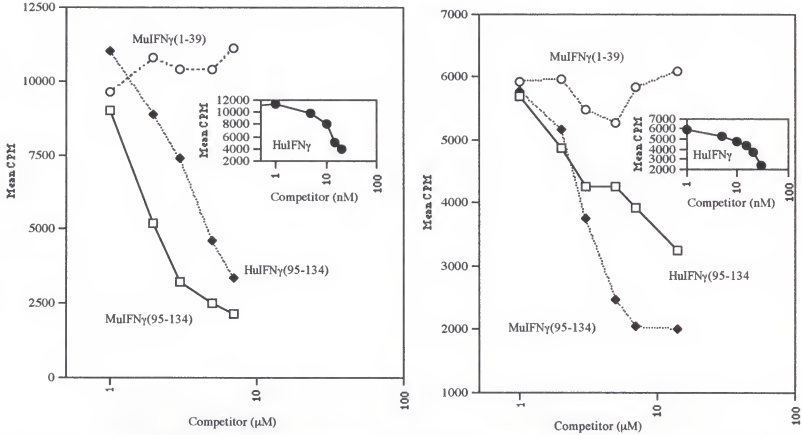


Figure 4. Dose response of HuIFNγ, and HuIFNγ peptides on ^{125}I-HuIFNγ binding to HuIFNγR and MuIFNγR. Binding of ^{125}I-HuIFNγ to solid-phase HuIFNγR cytoplasmic domain protein (Panel A) or the soluble MuIFNγR (Panel B) was performed as described in Figure 2. The competitors were unlabeled HuIFNγ (filled circle; insert graphs), HuIFNγ(95-134) (filled diamond), MuIFNγ(95-133) (empty square), and MuIFNγ(1-39) (empty circle). Binding in the absence of competitor was 7,000 CPM and 11,031 CPM on the soluble MuIFNγR and HuIFNγR cytoplasmic domain, respectively. Data represent the mean of triplicate wells.

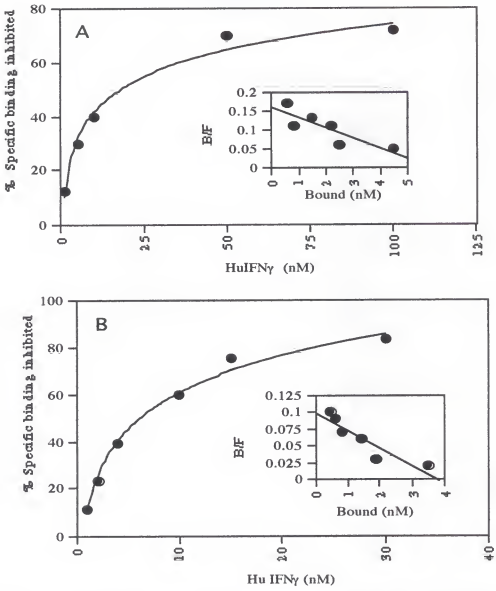


Figure 5. Scatchard analysis of the binding of HuIFNγ to the HuIFNγR cytoplasmic domain protein and the soluble MuIFNγR.

Scatchard plots (insets) and K_d values were obtained using the EBDA program for "cold" saturation binding data (19-22). Saturation binding of unlabeled huIFNγ to the HuIFNγR cytoplasmic domain (Panel A) and soluble MuIFNγR (Panel B) were determined by incubating increasing concentrations of unlabeled huIFNγ with 10 nM ^{125}I-HuIFNγ in microtitier plate wells absorbed with the receptor as in Figure 2. Control binding was determined in the absence of unlabeled HuIFNγ. Binding inhibition was calculated by subtracting the binding at the various concentrations of unlabeled HuIFNγ from the specific binding (total binding in the absence of unlabeled HuIFNγ- nonspecific binding determined at 800 nM unlabeled HuIFNγ) and expressed as percent of control binding inhibited (100%=38,000±4,000 cpm for HuIFNγR, Panel A; and 30,000±4,000 cpm for muIFNγR , Panel B). Data represent the mean of triplicate wells. Scatchard plots of binding data (inserts). B, bound; F, free.

Analysis of receptor saturation was performed using a standard "cold" saturation experiment (McPherson and Summers, 1983; McPherson, 1985) as shown in Figure 5A and 5B. Computer analysis of the binding data was used to obtain a scatchard plot (Figure 5A and 5B insets) and K_d values (McPherson, 1983; McPherson, 1985). The K_d of HuIFNγ for the HuIFNγR cytoplasmic domain protein (3.7×10^{-8} M) and the MuIFNγR cytoplasmic domain (7.2×10^{-8} M) were similar. The K_d of MuIFNγ for the MuIFNγR (5.5×10^{-9} M) was used as an experimental control for K_d determinations in solid-phase binding assays (Data not shown). This K_d was similar to that previously determined for MuIFNγ on MuIFNGR expressing cells (Kumar et al., 1989; Gray et al., 1989; Cofano et al., 1990; Fernando et al., 1991), and reflects high affinity binding to the extracellular domain of the receptor. The binding affinity of HuIFNγ for the cytoplasmic domain of the HuIFNGR α chain is similar to that of simian virus 40 (SV40) NLS binding to the nuclear transporter importin (Hubner et al, 1997). Thus, IFNγ binding to the receptor cytoplasmic domain is of sufficient affinity for possible nuclear translocation.

The site of interaction of IFNγ on the HuIFNGR cytoplasmic domain involves the receptor region adjacent to the cell membrane from the cytoplasmic side as indicated by the specific inhibition of ^{125}I-HuIFNγ binding to the receptor by receptor peptides HuIFNGR (252-291) and MuIFNGR (253-287) (Figure 6A). HuIFNGR (102-130), which is involved in the extracellular binding of HuIFNγ, had no effect on cytoplasmic binding. Similar inhibition patterns were observed for ^{125}I-huIFNγ binding to MuIFNGR (Figure 6B). Unlabeled HuIFNGR cytoplasmic domain protein (Figure 7A) and MuIFNGR (Figure 7B) were similar in their ability

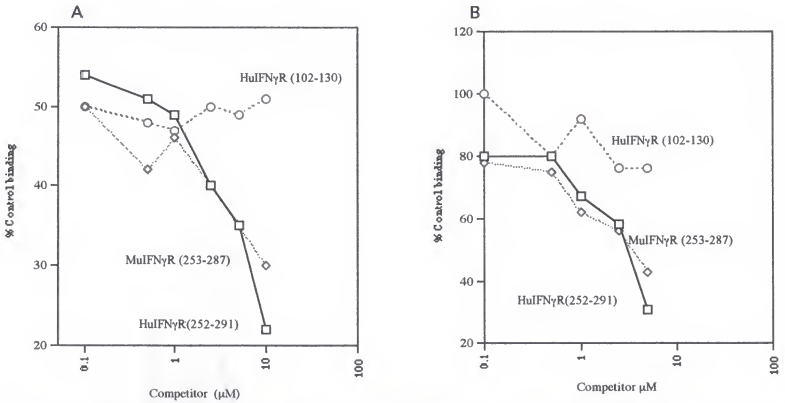


Figure 6. Effect of MuIFNγR peptide dosage on ^{125}IHuIFNγ binding to receptors. The soluble muIFNγR (10 ng) (Panel A) or the HuIFNγR cytoplasmic domain protein (10 ng) (Panel B) were absorbed to the wells of microtiter plates for 18 h at 4°C. ^{125}IHuIFNγ (5 nM) was incubated with the receptor absorbed to the plate in the absence (control) or presence of increasing concentrations of competitors. The competitors were HuIFNγR (252-291)(square), HuIFNγR (102-130)(circle), and MuIFNγR (253-287)(diamond). Specific binding (total binding - nonspecific binding of BSA at various concentrations) is expressed as percent of control specific binding (100%=3700 ±250 cpm for huIFNγR, Panel A; and 4200 ± 300 cpm for muIFNγ, Panel B). Data represent the mean of triplicate wells.

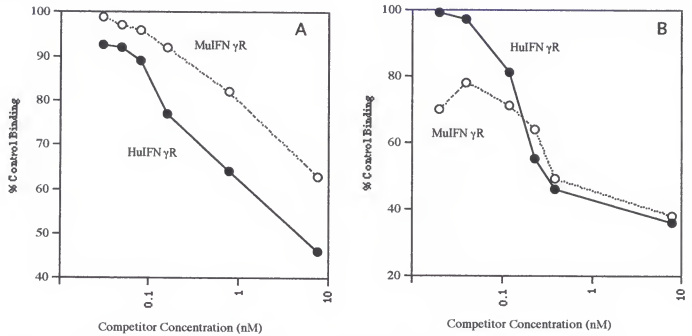


Figure 7. Effect HuIFNγR cytoplasmic domain protein and HuIFNγR on ^{125}I-HuIFNγ binding to receptors. HuIFNγR cytoplasmic domain protein (10 ng) (Panel A) or the soluble MuIFNγR (10 ng) (Panel B) were absorbed to the wells of microtiter plates for 18 h at 4^0C. ^{125}I-HuIFNγ (5 nM) was incubated with the receptor absorbed to the plate in the absence (control) or presence of increasing concentrations of competitors. The competitors were: HuIFNγR protein (filled circle), and MuIFNγR (open circle). Specific binding (total binding - nonspecific binding of BSA at various concentrations) is expressed as percent of control specific binding (100%=3700 ±250 cpm for HuIFNγR, Panel A; and 4200 ± 300 cpm for MuIFNγ, Panel B). Data represent the mean of triplicate wells.

to inhibit ^{125}I-HuIFNγ binding to the HuIFNGR cytoplasmic domain. Thus, receptor and receptor peptide competitions show that cytoplasmic binding of IFNγ occurs at corresponding sites for HuIFNGR and MuIFNGR, and this binding is species non-specific.

Differential Nuclear Localization of the α and β Subunits of the IFNGR Complex After Activation by IFNγ

Human WISH cells express a large number of IFNγ receptors

The binding of IFNγ to receptors on the IFNγ-sensitive human WISH cell line was characterized by studying the binding of ^{125}I-labeled IFNγ to these cells at 4°C. Binding studies were performed using standard "cold competition" experiments, and data analyzed using the LIGAND program (McPherson, 1985). As shown in Figure 8, a K_d of 4.78 x 10^{-9} M was determined for the binding of IFNγ, confirming high-affinity receptor sites for IFNγ on these cells. Approximately 100,000 high-affinity binding sites were estimated for IFNγ on these cells. These values are very similar to that reported previously by others (Sarcar et al., 1984). The large number of binding sites, and hence receptor molecules, on these cells provided us with a system for easily monitoring receptor movement by immunofluorescence and other techniques.

IFNγ-dependent selective nuclear translocation of IFNGRα versus IFNGRβ in human WISH cells

We have previously shown that the full-length human IFNγ is translocated to the nucleus (Subramaniam et al, 1998), using standard nuclear import assays.

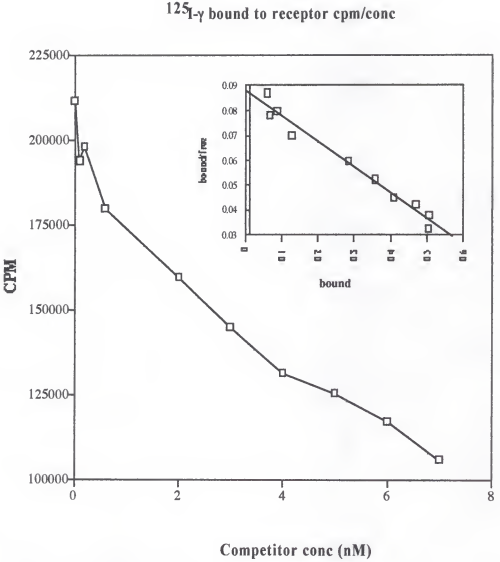


Figure 8. WISH cells express a high number of IFNγ receptor molecules. Binding of ^{125}I-IFNγ to WISH cells was assayed as described in the "Materials and Methods". Nonspecific binding, determined as binding in the presence of a 1000-fold excess of unlabeled IFNγ, was less than 18%. Data were analyzed using the LIGAND computer program to determine the binding constants. Values for points on the abscissa and ordinate were obtained from the above analysis and have been replotted here.

Human IFNγ utilizes components of the Ran/importin pathway similar to murine IFNγ (Subramaniam et al, 1998), and nuclear import of human IFNγ can be competed by both the NLS in murine IFNγ and the prototypical NLS in the SV40 T antigen (Subramaniam et al, 1998). The C-terminus of human IFNγ, like murine IFNγ, contains a nuclear localization sequence, ^{128}KRKR^{131}, in a highly conserved region (amino acids 95-134) in its C-terminus that overlaps, in its position, with the NLS of murine IFNγ. As has previously shown for the NLS of murine IFNγ, a peptide containing this human IFNγ NLS is able to target a heterologous protein for nuclear import, when employed in standard nuclear import assays using digitonin permeabilized HeLa cells. The nuclear import mediated by the human IFNγ NLS occurs in an energy-dependent fashion requiring GTP, and is strictly dependent on the addition of cytosolic factors. These data will be illustrated later within this dissertation. Thus, human IFNγ is targeted to the nucleus via a simple polybasic NLS that is similar to that in murine IFNγ.

Using human IFNγ on WISH cells, which do not produce IFNγ, the effects of IFNγ stimulation on the nuclear localization of IFNγ receptor subunits were determined. Cells treated with human IFNγ at various times were fixed, permeabilized and fluorescently stained using antibodies to IFNGRα, IFNGRβ and STAT1α, either alone or in combination, to monitor the localization of these proteins following receptor stimulation. Specificity of the antibodies was verified by incubation in the presence of excess of the peptide antigens used to generate the antibodies. Excess of the antigen was able to completely inhibit immunofluorescence staining (data not shown).

In these experiments, cells were simultaneously stained for the individual receptor subunits (Texas Red) and STAT1α (FITC) so that we could monitor both molecules in the same cell. Further, since STAT1α is well characterized with respect to its nuclear localization, double-staining experiments allowed us to verify the competence of these cells for IFNγ mediated receptor activation. As can be seen in Figure 9, in untreated cells both the IFNGR subunits (IFNGRα: Figure 9A; IFNGRβ: Figure 9B) essentially showed a diffuse localization on the cell. As expected, STAT1α was also present throughout the cytoplasm in untreated cells. It should be noted, however, that labeling which may appear nuclear is actually labeling of those receptors above the nucleus. Treatment of cells for 30 min with IFNγ altered the cellular distribution profile of IFNGRα and STAT1α. In both Figures 9A and 9B, in cells treated with IFNγ, STAT1α was localized in the nucleus. The pattern seen for STAT1 with respect to 2D immunofluorescence images and immunoprecipitation are very similar to what other published reports have shown for STAT1 in IFNγ treated cells (Haspel et al., 1996; Koster and Hauser, 1999), in which immunofluorescence images show a particularly strong nuclear presence of STAT1, compared to immunoprecipitations. In Figure 9A, IFNγ treated cells stained for the IFNGRα subunit showed that IFNGRα was also translocated to the nucleus. In marked contrast in Figure 9B, in IFNγ treated cells the IFNGRβ showed the same diffused profile as seen in untreated cells. These data show that the while IFNGRα, like STAT1α, is translocated to the nucleus, IFNGRβ is not. Thus, these data show for the first time that the IFNGRα and IFNGRβ

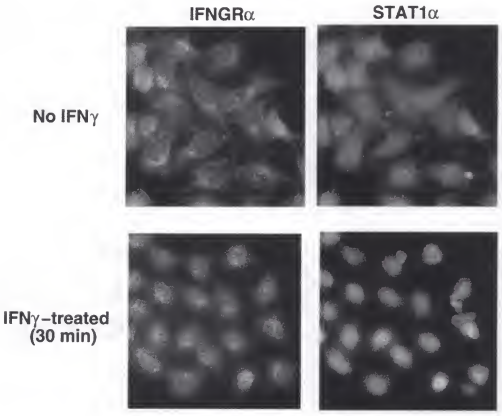


Figure 9. IFNγ treatment of WISH cells induces the nuclear translocation of the IFNGRα subunit, but not that of IFNGRβ. (**A**). WISH cells were either left untreated (No IFNγ) or treated with IFNγ as indicated for 30 min, and the cells fixed and immunofluorescently stained simultaneously with antibodies to IFNGRα (rabbit anti-IFNGRα) and STAT1α (goat anti-STAT1α). (**B**). Cells treated as in (A) were doubly stained with antibodies to IFNGRβ (rabbit anti-IFNGRβ) and STAT1α (rabbit anti-Stat1α). In immunofluorescence experiments, antibodies developed in rabbits were generally found to give stronger signals than other antibodies.

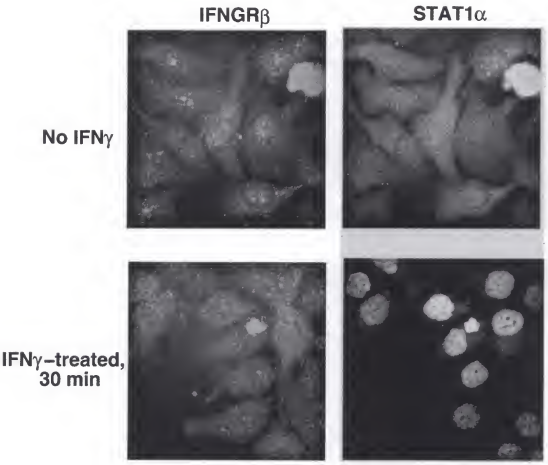


Figure 9 continued:

subunits of the receptor complex are differentially localized within the cell following receptor-mediated endocytosis.

To better characterize the localization of IFNGRα, we used deconvolution microscopy to render a 3D reconstructed image of IFNGRα and STAT1α staining in WISH cells before and after IFNγ treatment. Multiple image sections were made vertically through the plane of the cells and recorded, and image sections deconvolved. Deconvolved images were then merged to give Figure 10A. Figure 10B shows the same cells, except that in this case only a section 0.2 μm thick through the plane of the nucleus has been shown, projected at 0° and 90° angles. The latter figure (Figure 10B), shows that in the plane of the nucleus both IFNGRα and STAT1α are confined to the nucleus only in IFNγ-treated cells, confirming their nuclear localization following IFNγ treatment.

In these experiments, we have used STAT1α as a marker for nuclear translocation of the IFNγ receptor subunits. Although the relative fates of the two receptor subunits can be inferred by comparison of the two receptor subunits with respect to STAT1α translocation, a direct comparison of the two molecules within the same cells following IFNγ stimulation would be more definitive. Thus, cells were stained simultaneously for IFNGRα and IFNGRβ subunits before and after IFNγ stimulation. As can be observed in Figure 11, when examined within the same cells, IFNGRα chain shows nuclear accumulation subsequent to IFNγ stimulation while there is no observable change in the IFNGRβ chain. Thus, these data further confirm that IFNγ treatment of cells leads to the selective nuclear translocation of IFNGRα subunit.

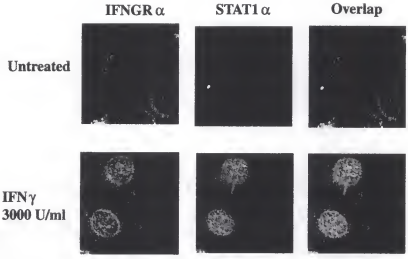


Figure 10. IFNγ treatment of cells induces the nuclear translocation of the IFNGRα along with STAT1α. 3D volume reconstruction of IFNGRα and STAT1α localization in WISH cells either left untreated, or treated with IFNγ (3000 units/ml). (**A**). After immunofluorescence images of cells were obtained through 64 image sections on a deconvolution microscope. After deconvolution of the stack, deconvolved sections were merged to render a 3D reconstruction of the cells, as shown. All image processing was done using DeltaVision (Applied Precision, Issaquah, Washington) software attached to a Silicon Graphics workstation. (**B**). 3D reconstruction as in (A) except that only 2 image sections from the stack, above and below the best focal plane through the nucleus of the cells (a 0.2 μm displacement each along the Z-axis), were merged. The resulting nuclear images have been projected by rotation at 0° (top panels) and 90° (bottom panels) along the Y-axis. Note that in the plane of the nucleus in IFNγ treated cells staining is still largely confined to the nucleus, unlike in untreated cells.

IFNγ-treated:

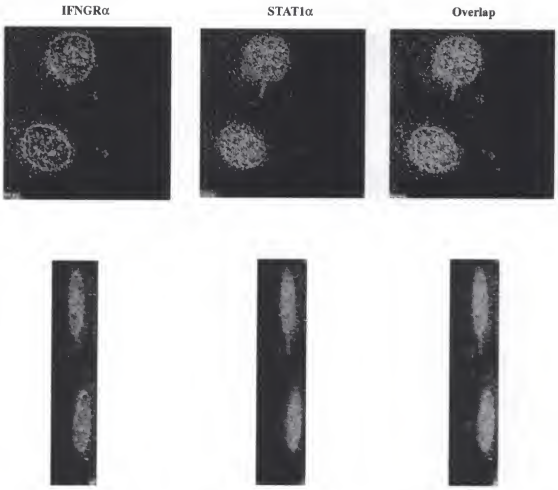


Figure 10 continued:

Untreated:

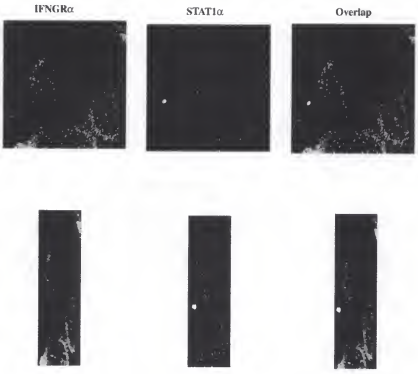


Figure 10 continued:

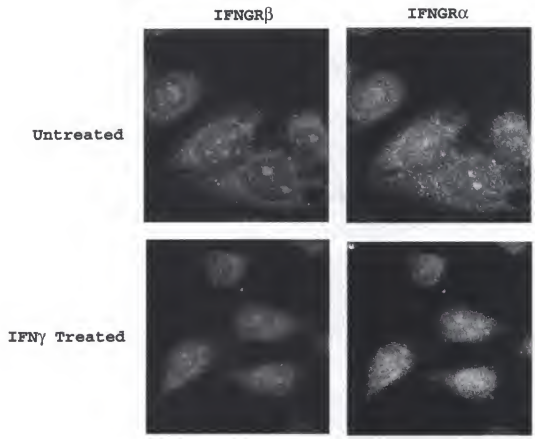
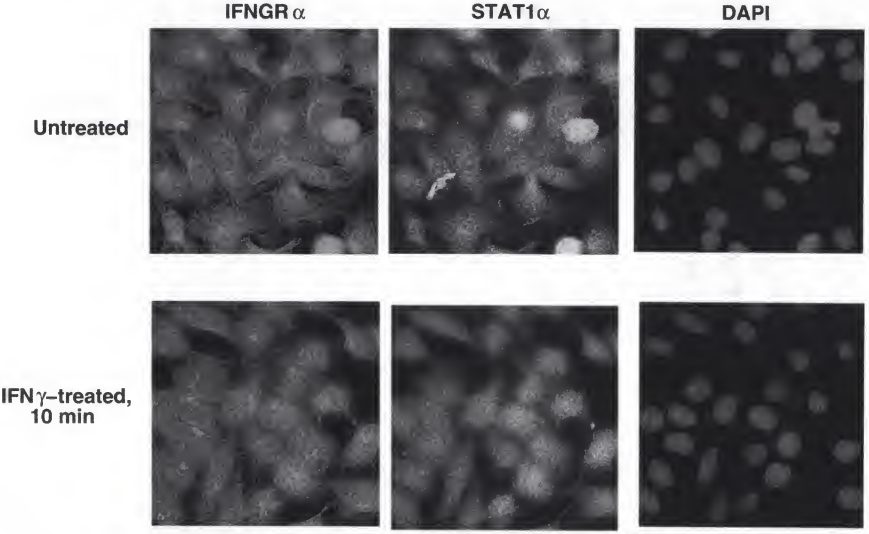


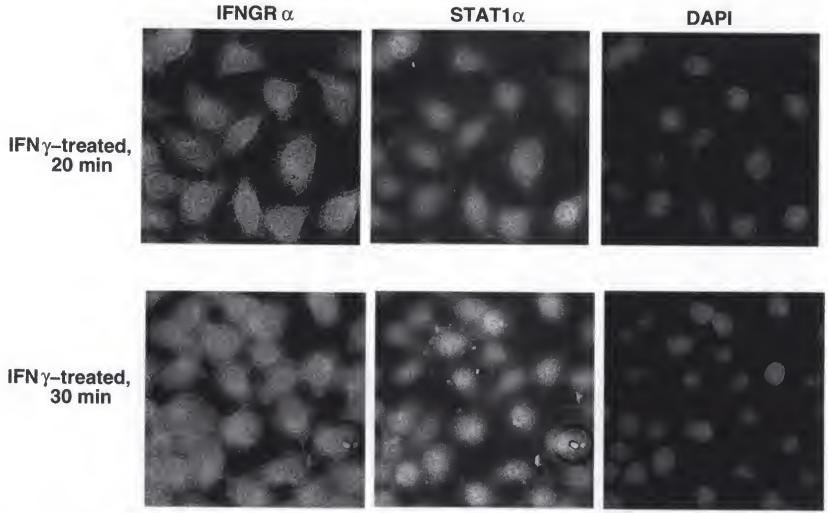
Figure 11. Differential nuclear localization of IFNGRα and IFNGRβ subunits as visualized within the same cells. Cells were treated as in Figure 9 with the exception of the antibody labeling procedure. Cells were first stained for the IFNγRβ chain antibodies . The cells were then stained with FITC-conjugated IFNγRa antibodies.

IFNGRα and STAT1α co-localize to the nucleus after IFNγ activation in a time- and dose-dependent fashion.

Time-course experiments of IFNGRα and STAT1α translocation were next performed in IFNγ-treated cells that were simultaneously stained for IFNGRα (Texas red) and STAT1α (FITC). At the same time, DAPI, a dye that stains nuclear DNA, was used to delineate the nuclear volume in the cells. As can be seen in Figure 12, over time the IFNGRα subunit showed increasing nuclear accumulation, demonstrating a time-dependent nuclear translocation of this subunit in these cells. The translocation was discernable within 10 min of stimulation of cells with IFNγ, peaked at 20 min, and started to decline thereafter, reflecting the exit of IFNGRα from the nucleus (Figure 12A). This translocation of IFNGRα was closely paralleled by the nuclear accumulation and nuclear exit of STAT1α in these same cells, and the pattern of staining for STAT1α appeared to overlap the pattern of staining for IFNGRα at each time point observed. A quantitative analysis of the nuclear fluorescence staining for IFNGRα and STAT1α within individual cells (Figure 12B) shows that rates of entry and exit of IFNGRα and STAT1α are coincidental, and that a constant ratio of IFNGRα to STAT1α is maintained throughout their traverse of the nucleus at any given time. These data suggest that the nuclear localization of STAT1α and IFNGRα may be linked. It is known that IFNγ is internalized via receptor mediated endocytosis involving complexation with the cell surface receptors. It is this ligand/receptor complex which is then internalized. Therefore, the receptor subunits which are undergoing changes in

Figure 12. Time-course analysis of the nuclear translocation of IFNGRα and STAT1α in WISH cells treated with IFNγ. (**A**). Cells were either left untreated or treated with IFNγ for the indicated time periods, as labeled in the Figure. Cells were then fixed and stained immunofluorescently with antibodies to IFNGRα (rabbit anti-IFNGRα) and STAT1α (goat anti-STAT1α). Nuclei in these doubly stained cells were then stained with DAPI to delineate the nuclear volume. (**B**). Quantitation images presented in (A) as described in 'Materials and Methods'. The average fluorescence ratio Fn/Fc for each field shown is plotted against time of stimulation with IFNγ.


IFNGR α
STAT1α
DAPI
Untreated
IFNγ–treated,
10 min


IFNGR α
STAT1α
DAPI
IFNγ–treated, 20 min
IFNγ–treated, 30 min

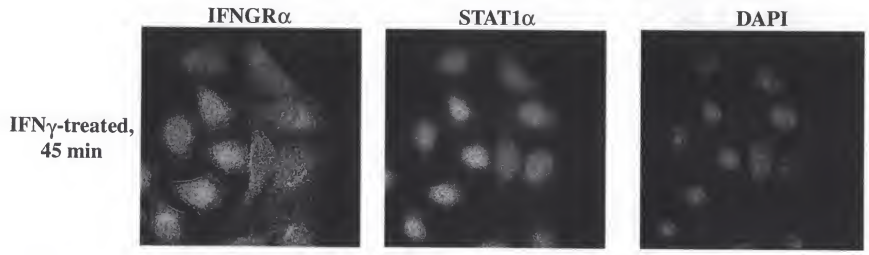


Figure 12 continued:

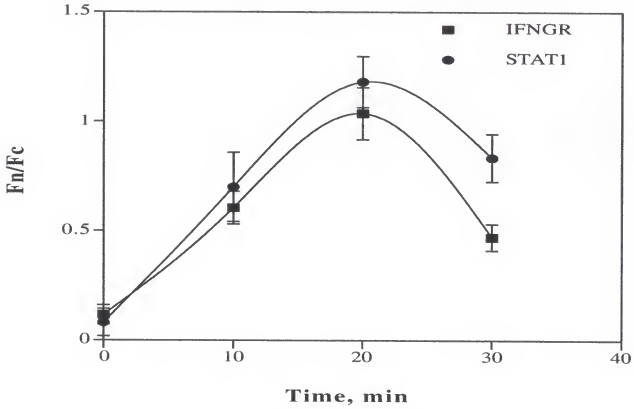
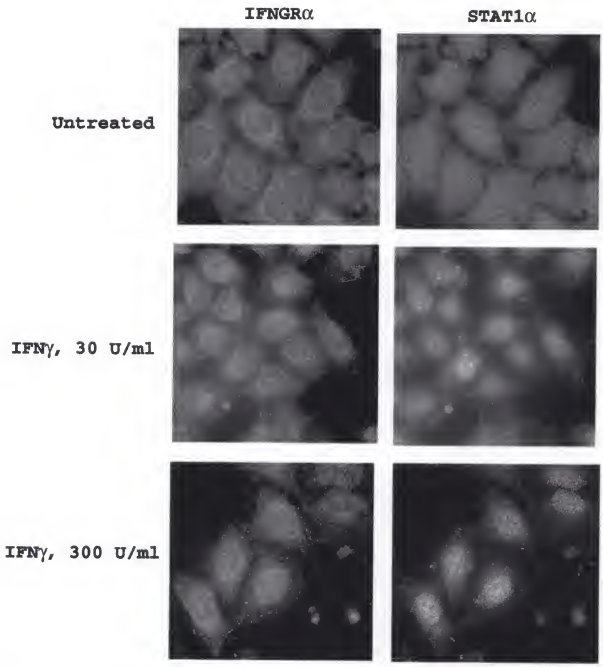


Figure 12 continued:

their localization are likely to be those on the surface which are directly exposed to the IFNγ. Again, no evidence was found for the nuclear accumulation of the IFNGRβ (data not shown). Thus, the IFNGRα subunit and STAT1α, but not IFNGRβ, were found to translocate to the nucleus with an apparent fixed stoichiometry and similar kinetics following IFNγ treatment of cells.

Dose response experiments examining the relationship of the nuclear translocation of IFNGRα and STAT1α with respect to increasing concentrations (antiviral units) of IFNγ are presented in Figure 13. As can be seen in Figure 13A, the accumulation of IFNGRα and STAT1α increased in a dose-response fashion. Moreover, IFNGRα and STAT1α appeared to co-localize in the nucleus in a dose-dependent fashion. In these experiments, we found that the time taken for both IFNGRα and STAT1α to enter and exit from the nucleus was itself a direct function of the concentration of IFNγ. Higher concentrations of IFNγ resulted in maximal nuclear accumulation much earlier than the 30 min used in these experiments (data not shown). Quantitation of the images (Figure 13B) again confirmed that the relative amounts of IFNGRα and STAT1α present in the nucleus paralleled each other for each concentration tested. This is consistent with the conclusion drawn from the data in Figure 12 that the nuclear translocation of IFNGRα and STAT1α appear to be coupled. Further, since the amounts of IFNγ used were titrated as units of antiviral activity it appears that there is a close relationship between biological activity and the coupled activation and nuclear translocation of IFNGRα and STAT1α.

Figure 13. Dose-response analysis of the nuclear translocation of IFNGRα and STAT1α in WISH cells treated with IFNγ. (**A**). Cells were treated for 30 min at 37°C with the indicated concentrations of IFNγ and cells were then stained as in Figure 4, except that DAPI was not added. (**B**). Quantitation of images in Figure 5A was as for Figure 4, except that Fn/Fc was plotted against antiviral units of IFN added.


IFNGRα
STAT1α
Untreated
IFNγ, 30 U/ml
IFNγ, 300 U/ml

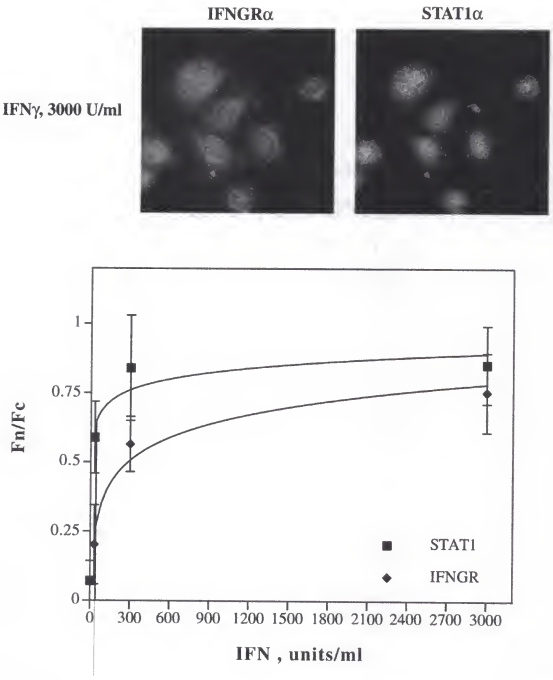


Figure 13 Continued:

Immunoprecipitation of isolated cytoplasmic and nuclear extracts confirm the selective nuclear accumulation of IFNGRα

To further confirm the differential nuclear localization of the IFNGR subunits, extracts from nuclei isolated from untreated and IFNγ treated cells were examined along with the corresponding cytoplasmic (including membrane) extracts by immunoprecipitation and immunoblotting with antibodies to IFNGRα, IFNGRβ, and STAT1α.

As can be seen in Figure 14A, in cytoplasmic extracts the IFNGRα subunit, consistent with its glycosylated nature, migrated as a diffuse band in both untreated and IFNγ treated cells. By contrast, IFNGRα was detected in nuclear extracts of only IFNγ stimulated cells (Figure 14B), consistent with the ligand-dependent accumulation of this subunit in the nucleus. Likewise, in these cells, STAT1α is also translocated to the nucleus only after IFNγ treatment (Figure 14E and 14F). Moreover, the time-course for the entry and exit of STAT1α parallels the time-course of IFNGRα. Thus, these data support the immunofluorescence experiments. The relative absence of IFNGRα and STAT1α in nuclear extracts of untreated cells, compared to their strong presence in cytoplasmic extracts, demonstrates that minimal cross-contamination of nuclear extracts occurred. Cross-contamination was also ruled out by comparing the specific activity of the cytoplasm-specific enzyme lactate dehydrogenase. No detectable activity was seen in nuclear extracts (see Materials and Methods).

Unlike the IFNGRα subunit, no accumulation of IFNGRβ chain was seen in the nuclei of IFNγ-treated cells in immunoblots comparing nuclear and cytoplasmic

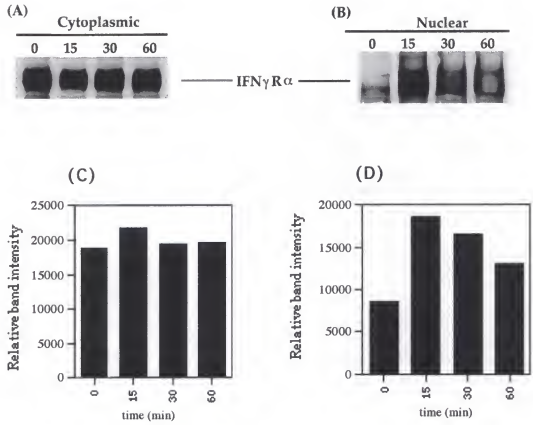


Figure 14. Immunoprecipitation of IFNGRα, but not of IFNGRβ, from nuclear extracts of IFNγ treated WISH cells. Cytoplasmic and nuclear extracts from WISH cells, either left untreated (0) or treated with IFNγ for 15 min (15), 30 min (30), 60 min (60) or 90 min (90), were prepared as described in "Materials and Methods". Extracts were immunoprecipitated and immunoblotted with antibodies to IFNGRα or STAT1α as follows: Cytoplasmic (A, C) and nuclear extracts (B, D) were immunoprecipitated and immunoblotted with antibodies to IFNGRα (A, B) and STAT1α antibodies. (E, F). Immunoblots for STAT1α from cytoplasmic extracts and nuclear extracts presented in (G) and (H) were probed with antibodies specific for Tyr^{701} phosphorylated STAT1α. (M). Cytoplasmic extracts were immunoprecipitated with antibodies to IFNGRα and analyzed by immunoblotting with antibodies to STAT1α. All antibodies were developed in rabbits. For these experiments equal amounts of protein were used within a given blot. Quantitation of the nuclear blots was also perfomed by densitometric analysis

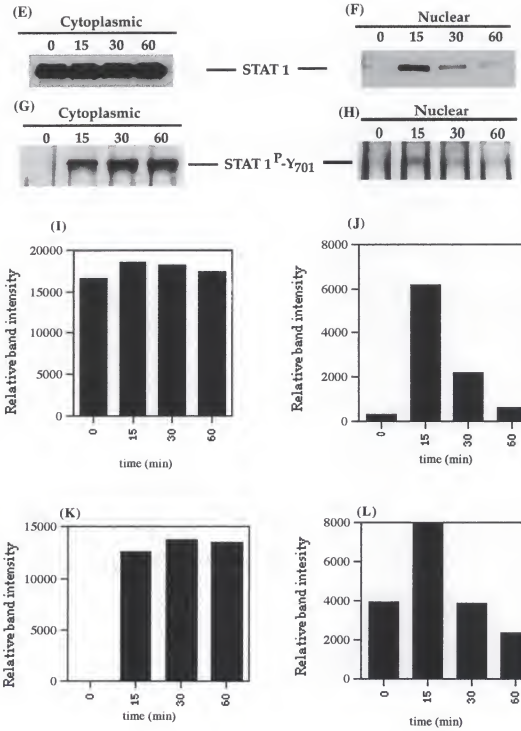


Figure14 continued:

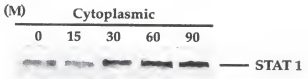
extracts (data not shown), although ligand-specific STAT1α accumulation in the nuclei of the same cells was again demonstrated (data not shown).

Figure 14G and 14H show the tyrosine phosphorylation status of STAT1α in the cytoplasm and the nucleus, respectively. The antibody used specifically recognizes STAT1α molecules tyrosine phosphorylated on Tyr^{701}, which is the only tyrosine residue that is phosphorylated in response to IFNγ (Shuai et al., 1992; Shuai et al., 1993). Tyrosine phosphorylation of the target Tyr^{701} residue of STAT1α could be detected both in the cytoplasm and nucleus. The level of nuclear tyrosine phosphorylation was consistent with the limited portion of the total cellular STAT1α that was present in the nucleus at any given time. In particular, in the nucleus the tyrosine phosphorylation profile of STAT1α (Figure 14H) mimicked that of the entry and exit of total nuclear STAT1α (Figure 14F), which in turn overlaps with the nuclear entry and exit of the IFNGRα chain (Figure 14B). Thus, the nuclear translocation of IFNGRα coincides specifically with the nuclear entry of STAT1α that is activated on the critical Tyr^{701} by recruitment to the IFNGRα chain. This raises the possibility that Tyr^{701}-activated STAT1α that is bound to the receptor IFNGRα may be translocated to the nucleus as a complex with IFNGRα.

The data in Figure 14F and 14H show that, under these conditions, we do not see sustained presence of tyrosine phosphorylated STAT1α in the nucleus even in the presence of continued activation by ligand (compare Figure 14E and 14G). This is consistent with the earlier studies (Haspel et al., 1996; Koster and Hauser, 1999). We also found the same to be true for the IFNGRα chain (compare Figure 14B and 14F). In the case of STAT1α this has been shown to be due to the dynamic

equilibrium, at the nuclear level, between the activation and nuclear translocation of STAT1α by JAK kinases and the deactivation of STAT1α by a nuclear phosphatase (Haspel et al., 1996). This is manifested as an appearance in the nucleus of total (serine plus tyrosine) phosphorylated STAT1α following activation, and the subsequent rapid exit of nuclear STAT1α following dephosphorylation (Haspel et al., 1996). The exit phase from the nucleus is accompanied by accumulation of total (serine plus tyrosine) phosphorylated STAT1α in the cytoplasm (Haspel et al., 1996), the significance of which is not known, but does suggest that in the continued presence of ligand, JAK activation of STATs occurs over a time course that probably exceeds the requirements for immediate-early gene activation.

The dynamics of IFNGRα and STAT1α association in the cytoplasm was also examined in the above context. As shown in Figure 14 M, cytoplasmic extracts of untreated and IFNγ treated cells were immunoprecipitated with antibodies to IFNGRα and following Western blotting probed with antibodies to STAT1α. Low levels of STAT1α were complexed with IFNGRα in the cytoplasm (Figure 14 M) at times when the levels of nuclear accumulated STAT1α (Figure 14 F) and nuclear IFNGRα (Figure 14 B) were relatively high, consistent with the rapid transport of IFNGRα and STAT1α to the nucleus, possibly via a complex of IFNGRα and STAT1α. Also, increased STAT1α/IFNGRα complexes were seen during the slow cytoplasmic accumulation phase of tyrosine phosphorylated STAT1α that follows the exit of both IFNGRα and STAT1α from the nucleus. Thus, the immunofluorescence and immunoprecipitation data suggest that the IFNGRα subunit may play a role in the transport of STAT1α to the nucleus. However, the



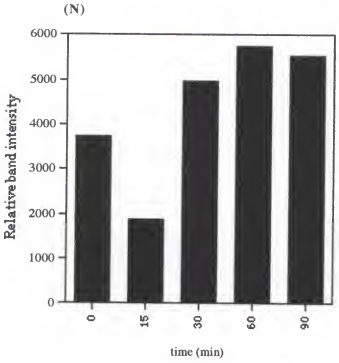


Figure 14 continued:

two molecules appear to dissociate in the nucleus since STAT1α did not co-immunoprecipitate with the IFNGRα chain in the nucleus (data not shown).

The findings reported here showing association of IFNγ activated IFNGRα and STAT1α up to nuclear translocation may appear at odds with models of IFNγ signaling, where STAT1α is postulated or shown to dimerize, dissociate from the receptor, and then undergo nuclear translocation (Bach et al., 1997; Pestka et al., 1997). Our data suggest that some, but not all, STAT1α remains associated with the IFNGRα subunit upon IFNγ stimulation and probably undergoes nuclear translocation associated with this subunit. These findings do not preclude the presence of free STAT1α homodimers as reported by others. Our data do, however, provide one approach for determination of the mechanism of nuclear translocation of STAT transcription factors.

The C-terminal region of IFNγ has biological significance

A number of earlier studies have shown that truncations in the C-terminus of IFNγ that destroy the polybasic region, which we have identified as a NLS, lead to drastic loss in the biological properties of IFNγ (Arakawa et al., 1986; Dobeli et al., 1988; Arakawa et al., 1989; Wetzel et al., 1990; Lundell et al., 1991; Slodowski et al., 1991). These studies suggest that the NLS is critical for the biological activity of IFNγ. As a starting point to analyzing the role in signal transduction of the NLS in the C-terminus of IFNγ, we re-examined one such mutant, IFNγ (1-123), that is deleted from residues 124 (see Table 3 for sequences) onwards including the IFNγ NLS. To determine the structural and functional properties of IFNγ (1-123), we

Table III . Sequences referred to in this study.

Hu IFNγ C-terminus[a]	------^{91}KRDDFEKLTNYSVTDLNVQRKAIHELIQVMAELSPAA**KTGKRKRS**QMLFRGRRASQ
Hu IFNγ(1-123) C-terminus[a]	------^{91}KRDDFEKLTNYSVTDLNVQRKAIHELIQVMAEL^{123}
IFNγ(95-134) (human)	FEKLTNYSVTDLNVQRKAIHELIQVMAELSPAAKTG**KRKR**
IFNγ(95-133) (mouse)	MSIAKFEVNNPQVQRQAFNELIRVVHQLLPESSL**RKRKR**
IFNγ(95-125) (mouse)	MSIAKFEVNNPQVQRQAFNELIRVVHQLLPE
IFNγ (122-132) (human)	AA**KTGKRKRS**

[a] The sequences are shown only for the relevant C-terminal portions of the intact IFNs. Sequences are derived from the mature form of IFNγ. NLS sequences are in bold.

compared it with wild-type IFNγ in in vitro binding assays and antiviral assays. Fig. 15 shows the Scatchard analysis of a standard "cold" saturation experiment on binding of IFNγ(1-123) to WISH cells and its comparison with wild-type IFNγ (Green et al., 1998). Scatchard analysis of the binding showed that IFNγ(1-123) bound to receptors on WISH cells with a K_d that was very similar to that of wild-type IFNγ. Thus, IFNγ(1-123) was just as competent as wild-type IFNγ in binding to receptors on intact cells. Deletion of the C-terminal amino acids, including the NLS, from IFNγ did not significantly affect the affinity of IFNγ(1-123) for the receptor, showing that the C-terminal amino acids in general, and the NLS in particular, do not contribute significantly to high–affinity binding to the receptor complex. This conclusion is further supported by recent studies using surface plasmon resonance to address the binding of IFNγ(1-124) to the extracellular domain of IFNGRα (Sadir, et al., 1998), and by data from the X-ray crystal structure of the complex between IFNγ and the extracellular domain of IFNGRα (Walter et al., 1995).

When compared in antiviral assays, however, IFNγ(1-123) was found to be drastically reduced in its biological activity. IFNγ(1-123) had less than 0.5% of the specific antiviral activity of wild-type IFNγ when compared on the same WISH cells (data not shown). This is consistent with the earlier functional studies on C-terminal deletion mutants (Arakawa et al., 1986; Dobeli et al., 1988; Arakawa et al., 1989; Wetzel et al., 1990; Lundell et al., 1991; Slodowski et al., 1991). Thus, while the NLS-containing C-terminal region does not seem to contribute significantly to high-affinity interactions for binding to the receptor complex on WISH cells, it

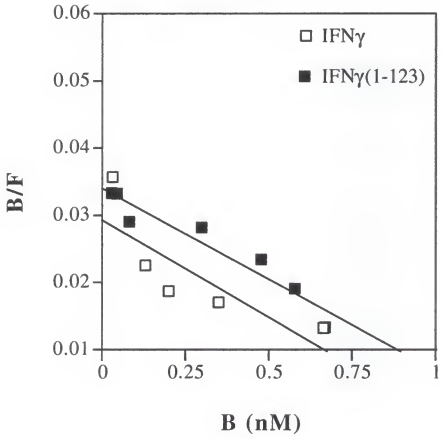


Figure 15. Comparison of binding constants for IFNγ and IFNγ(1-123) on WISH cells. Binding was evaluated in a standard "cold" saturation experiment using ^{125}I-IFNγ (5 nM). Data were analyzed using the LIGAND computer program, and data from Scatchard analysis of binding have been replotted here. Samples were run in triplicate. Specific binding of ^{125}I-IFNγ, determined in the presence of a 100-fold excess of unlabeled IFNγ, was found to be > 80%.

appears to play a crucial role in the ability of IFNγ to induce a biological response in these cells. The possibility, however, that this region is involved in low-affinity interactions with the extracellular domains of the receptor cannot be ruled out.

Since nuclear translocation of STAT1α is dependent on phosphorylation of STAT1α by JAK1 and JAK2, we used nuclear translocation of STAT1α as a marker of receptor activation. Cells were treated with IFNγ(1-123) and nuclear localization of STAT1α was compared with that of wild-type IFNγ. As can be seen in Fig. 16, IFNγ(1-123) was impaired in its ability to induce STAT1α nuclear translocation, compared to IFNγ. Thus, the deletion of the NLS in IFNγ is coincident with the loss of ability to induce the activation and nuclear translocation of STAT1α. Since STAT1α's presence in the nucleus is required for biological activity this is consistent with the poor biological activity of IFNγ(1-123).

Coprecipitation of STAT1 and IFNγ with importin α.

Nuclear transport of STAT1α occurs through the interaction of activated STAT1α with the importin-α analog Npi-1. Thus, since IFNγ was found complexed with STAT1α we determined whether IFNγ was complexed with Npi-1. To further gain insight into the role of the C-terminal NLS of IFNγ in this complexation and regulation, we also used the human deletion mutant IFNγ(1-123) that lacks the NLS in similar studies and compared the results with wild-type IFNγ. ^{125}I-labeled human IFNγ and human IFNγ(1-123) were used to treat human WISH cells and lysates were immunoprecipitated with Npi-1 antibodies. As seen in Figure 17 (lower panel), wild-type IFNγ was recovered as a complex with Npi-1, however, the NLS-mutant IFNγ(1-123) was not found to be complexed with Npi-1. These data

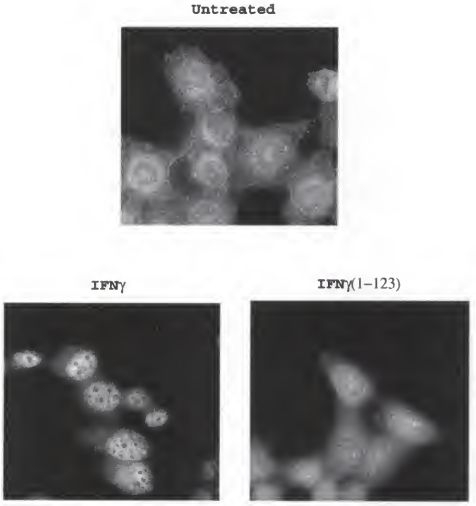


Figure 16. Deletion of the NLS in IFNγ inhibits nuclear translocation of STAT1α. WISH cells were treated for 30 min at 37°C with equal amounts (2.5 ng/ml) of IFNγ or IFNγ(1-123), or left untreated, as indicated, before being fixed and subjected to immunofluorescence staining for STAT1α localization using rabbit anti-STAT1α antibodies.

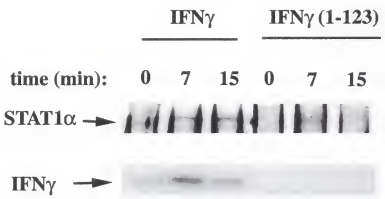
show that the NLS is required for complexation with Npi-1. When these complexes were examined for the presence of STAT1α (Figure 17, upper panel), STAT1α was found to co-immunoprecipitate in significant amounts only in cells treated with wild-type IFNγ. Thus, the interaction of the NLS of IFNγ with the importin Npi-1 is required for the formation of a stable complex between STAT1α and its nuclear transporter Npi-1. Npi-1 immunoprecipitated from cells treated with IFNγ (1-123) showed a low and transient signal for STAT1α, suggesting some complexation of STAT1α with Npi-1 could occur in a NLS-independent manner but this complex is weak and short-lived. These data strongly suggest that the NLS of IFNγ directly regulates the Npi-1-mediated entry of STAT1α into the nucleus.

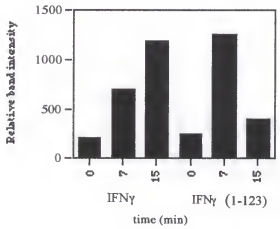
In the latter experiments, a small amount of phosphorylated STAT1α was detectable in the nucleus of cells treated with IFNγ (1-123) (data not shown) that coincided with the transient Npi-1:STAT1α complex seen in Figure 17 in similarly treated cells. The kinetics of this transport differed from that of wild-type IFNγ. This suggests that small amounts of STAT1α can translocate to the nucleus through other mechanisms. This may be related to the fact that a second weaker NLS in human IFNγ exists upstream of the one studied here (see also Bader and Witzerbin, 1994). It remains to be determined if this NLS can function similarly to that described here.

The carboxy terminus of HuIFNγ contains a functional NLS

The competence of the two putative nuclear localization sequences within the COOH-terminal domain of human IFNγ (HuIFNγ) was evaluated by testing its ability to mediate the nuclear import of a heterologous protein. This was performed

Figure 17. The formation of an Npi-1/STAT1α complex requires the IFNγ NLS. Human WISH cells were treated with 0.33 μg/ml each ^{125}I-IFNγ or ^{125}I-IFNγ(1-123), as indicated, for 7 min or 15 min at 37°C. Control cells were incubated with the appropriate ligands at 4°C . Cells were lysed and immunoprecipitated with antibodies to Npi-1. IFNs were followed by autoradiography, while STAT1α was detected by immunoblotting with anti-STAT1α antibodies. Quantitation of STAT blots was performed by densitometric analysis.


IFNγ
IFNγ (1-123)
time (min): 0 7 15 0 7 15
STAT1α
IFNγ


Relative band intensity
1500
1000
500
0
0 7 15 0 7 15
IFNγ
IFNγ (1-123)
time (min)

using the standard *in vitro* nuclear transport assay in digitonin permeabilized human HELA cells (Adam et al., 1992).

In this study we used as a substrate a peptide corresponding to amino acids 122-132 of HuIFNγ coupled to the heterologous autofluorescent APC for use in import assays. Figure 18 shows that essentially all of the Hela cells show nuclear accumulation of HuIFNγ (122-132). It also shows that this nuclear accumulation is inhibited at 4° and is dependent on reticulocyte derived cytosolic factors establishing the fact that this HuIFNγ (122-132) is capable of mediating the nuclear import of a heterologous protein coupled to it.

The transport of large molecules across the nuclear pore is a strictly energy dependent process, dependent on both ATP and GTP. It has been shown that in the absence of these energy providing compounds a "nuclear rimming" pattern can be observed (Newmeyer and Forbes, 1988). Figure 19 shows that the import of HuIFNγ (122-132) into the nucleus is dependent on the addition of both ATP and GTP to the cytosolic extracts. The absence of ATP and the absence of GTP with the addition of the nonhydrolyzable analog GTPγS both result in the inability of HuIFNγ (122-132) to mediate the translocation of APC to the nucleus. In Figure 19 the documented "nuclear rimming" can be observed (in particular no GTP). Again, these data demonstrate that HuIFNγ (122-132) contains an NLS that functions in an energy dependant function similar to other nuclear import signals.

These data serve to demonstrate that the domain of HuIFNγ represented by HuIFNγ (122-132) contains an NLS capable of functioning in an energy dependent capacity similar to other nuclear import signals. HuIFNγ (122-132) is capable of

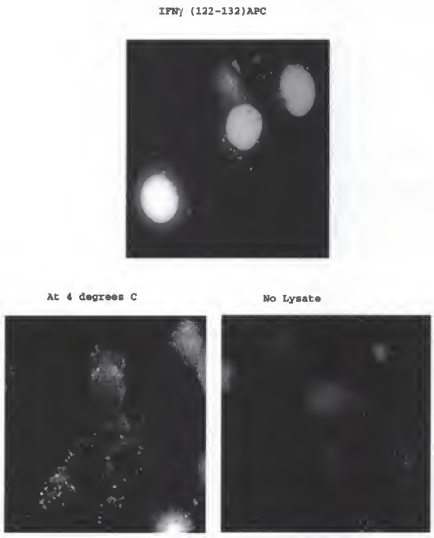


Figure 18. The peptide HuIFNγ (122-132) mediates the nuclear import of the heterologous protein APC. Digitonin permeabilized human HELA cells were incubated for the duration of the assay (30 min) with the complete import reaction mix containing HuIFN-γ (122-132)-APC as substrate at 30°C (top), or at 4°C (bottom left). The picture labeled "no lysate" depicts cells incubated with an import mixture devoid of reticulocyte lysate.

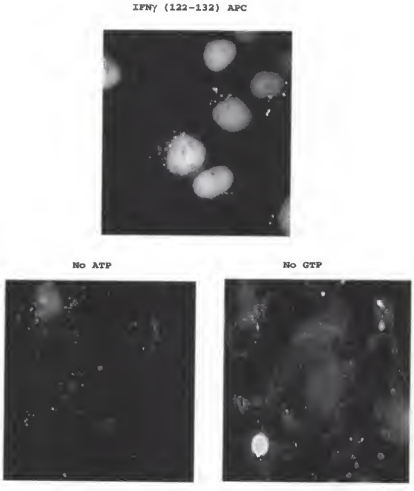


Figure 19. Nuclear import directed by HuIFN$_\gamma$ (122-132) is strictly energy dependent. Cells were incubated, as in Figure 18, either with complete import mixture or in the absence of ATP or GTP as described under material and methods.

mediating the nuclear import of a heterologous protein in an energy dependent manner.

The SV40 large T-antigen NLS is one of the best understood and established simple polybasic NLS sequences. It has been shown that carboxy- terminus of murine interferon gamma contains a polybasic NLS sequence capable of competition with the SV40 NLS for the cellular machinery necessary for nuclear import. We therefore wanted to see if the same could be said for the human counterpart. Figure 20 clearly shows that excess SV40 NLS peptide is capable of completely inhibiting the import of HuIFNγ (122-132) conjugated APC into the nucleus. These data demonstrate that HuIFNγ (122-132) utilizes the same nuclear import pathway used by SV40 large T-antigen.

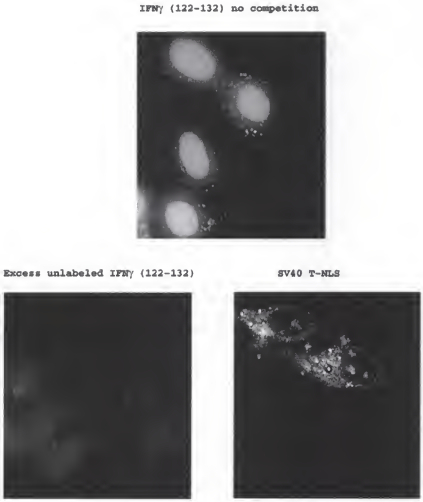


Figure 20. Nuclear import directed by HuIFNγ (122-132) is sequence-specific and inhibited by the SV40 T-NLS. Import reaction mixtures containing the substrate IFNγ (122-132)-APC were incubated either in the absence of competitor peptides or in the presence of the cognate peptide or the SV40 T-NLS peptide. Competitor peptides were incubated at a 600-fold molar excess, with respect to the substrate HuIFNγ (122-132)-APC, in the reaction mixture 5 min before addition of the substrate.

CHAPTER 4

DISCUSSION

In collaboration with others I have shown that after the receptor-mediated endocytosis of IFNγ, the IFNγ molecule and the IFNγ receptor play an active role in cellular signaling. Using the subcloned and expressed cytoplasmic domain of the HuIFNGRα without its high affinity extracellular domain and the soluble MuIFNGRα, we correlated the previously observed biological activity of internalized IFNγ and IFNγ peptides and relative affinity for the cytoplasmic domain.

Because glycosylation can affect protein solubility, structure, antigenicity, and the observed M_r, we evaluated our protein for oligosaccharides. Treatment to remove N-linked carbohydrates did not affect the observed M_r. In addition, our observed M_r of 43 KDa is consistent with the M_r observed for the intact, deglycosylated HuIFNγR isolated from placental tissue (Calderon et al., 1988). Thus, we concluded that the difference in the predicted M_r of 30 KDa and the M_r of 43 KDa determined for the receptor cytoplasmic domain expressed in *Pichia* reflected a difference in the predicted and observed M_r seen with the intact receptor α chain (Aguet et al, 1988; Calderon et al, 1988).

The species non-specific nature observed for HuIFNγ binding to the HuIFNγR and MuIFNγR cytoplasmic domains may be explained by the 88% amino acid sequence homology the receptors share in their membrane proximal cytoplasmic region. The site-specific binding of HuIFNγ, C-terminus HuIFNγ (95-134) and C-terminus MuIFNγ (95-133) to the membrane proximal region of each receptor cytoplasmic domain was confirmed by dose-dependent binding inhibition and subsequent saturation of the available binding sites with these ligands. The affinity constants for HuIFNγ binding to HuIFNGR and MuIFNGR cytoplasmic domains obtained from these assays, 3.7×10^{-8} M and 7.2×10^{-8} M, respectively, resembled those for SV40 large tumor antigen NLS binding to the nuclear import protein importin (Hubner et al., 1997). The similarity in K_d values suggest that the affinity with which HuIFNγ binds the cytoplasmic domain is sufficient to effect cytosolic and nuclear transport.

Past and present work in our laboratory has helped delineate a functional role for cytoplasmic domain binding in the biological response to IFNγ. Two of the three components known to be required for IFNγ signal transduction, tyrosine kinases JAK1 and JAK2 (Wilks et al., 1991; Harpur et al., 1992; Valazquez, 1992; Muller et al., 1993; Watling et al., 1993), bind to specific sites on the cytoplasmic domain of the IFNγR (Szente and Johnson, 1994; Szente el al., 1994). JAK1 and JAK2 then effect tyrosine phosphorylation of the third component, transcription factor STAT1, which translocates to the nucleus to initiate transcription of IFNγ inducible genes (Fu, 1992; Schindler et al., 1992; Schindler et al., 1992; Shuai et al., 1992; Decker et al., 1991; Igarashi et al., 1993; Greenlund et al., 1994, Farrar et al.,

1992). Thus, it appears that IFNγ, JAK1, JAK2, STAT and other components required for inducing the biological response attributed to IFNγ must associate directly or indirectly with the receptor cytoplasmic domain. We propose that this complex could then be translocated to the nucleus via a nuclear localization motif we have identified in the amino acid sequence of IFNγ (Subramaniam et al., 1998). Considered with the biological response elicited to IFNγ and C-terminal IFNγ peptides delivered to the cytoplasm by pinocytosis, liposomes or microinjection and the site specific binding of JAK1 and JAK2 in the cytoplasmic domain, our results establish a physiological connection between specific receptor-like binding of IFNγ to the cytoplasmic domain and IFNγ induced biological activity.

Having obtained this relationship between the cytoplasmic domain of the IFNγRα and IFNγ induced biological activity, we subsequently examined the activities of IFNγRα following IFNγ stimulation. Using the IFNγ receptor complex on intact cells, we have shown for the first time the selective internalization and nuclear transport of one subunit of a multimeric cytokine receptor complex, following ligand-dependent receptor activation. Among the two subunits of the IFNγ receptor complex, IFNGRα and IFNGRβ, only the IFNGRα subunit is selectively translocated to the nucleus in a ligand-dependent fashion. The nuclear translocation of IFNGRα is rapid, and occurs within the same time frame as the activation and nuclear translocation of the latent cytoplasmic transcription factor STAT1α associated with the receptor. This possibly suggests a role for the IFNGRα subunit in signal transduction events leading to immediate-early gene induction.

These studies strengthen the assumption that following ligand binding the IFNGRα chain is a central player in the transduction of the signal intracellularly. The IFNGRα chain has been recognized as the predominant high-affinity binding subunit for ligand. The intracellular domain of the IFNGRα subunit contains several elements involved in signal transduction. These include (i) a membrane proximal cytoplasmic binding site for the C-terminus of the ligand IFNγ -- this C-terminus region of IFNγ contains a functional NLS; (ii) a dileucine-containing membrane proximal domain required for receptor-mediated endocytosis (Farrar et al., 1991) that overlaps with the binding site for the NLS domain within the ligand (Szente et al., 1994; Szente and Johnson, 1994; Szente et al., 1995); (iii) a binding site for the tyrosine kinase JAK1 (Subramaniam et al., 1998; Farrar et al., 1991); (iv) a tyrosine phosphorylation motif for the docking of the transcription factor STAT1α (Bach et al., 1997; Pestka et al., 1997) to mediate its subsequent activation by the kinases JAK1 and JAK2, and (v) a ligand-induced co-operative binding site for JAK2 (Szente et al., 1995; Kotenko et al., 1995) which is in turn immediately proximal to the binding site for the C-terminal NLS domain of IFNγ (Szente et al., 1995). In contrast, the cytoplasmic domain of IFNGRβ only contains the site for the binding of JAK2 (Bach et al., 1997; Pestka et al., 1997), and JAK2 is constitutively attached to it (Bach et al., 1997; Pestka et al., 1997). Unlike the IFNGRα subunit, it is not phosphorylated in response to ligand binding. The attachment of JAK2 to the cytoplasmic side of IFNGRβ is, however, essential for bringing JAK2 into the receptor complex in close proximity to JAK1 and STAT1α to initiate the signal transduction events. The IFNGRβ subunit, thus, serves a

limited but indispensable structural role, that of providing a structural framework for the recruitment of JAK2 (Bach et al., 1997; Pestka et al., 1997). The specific, low-affinity but essential interaction of IFNγ with the extracellular domain of IFNGRβ is probably responsible for the transfer of JAK2 from IFNGRβ to IFNGRα. This is supported by the finding that while JAK2 can be found constitutively attached to IFNGRβ, JAK2 has been shown to co-immunoprecipitate with IFNGRα only after ligand binding (Kotenko et al., 1995), and is consistent with our findings that a co-operative binding site for JAK2 on the cytoplasmic domain of IFNGRα is induced by the interaction of the C-terminus of IFNγ at an immediately proximal site (Szente et al., 1995). Our studies thus suggest that while the IFNGRβ chain is required only for a limited but essential function, the IFNGRα continues to play a role in the further steps of signal transduction that may include the intracellular trafficking and nuclear delivery of signal components like STAT1α after their initial activation at the plasma membrane.

In this regard STAT1α has been demonstrated to undergo a cycle of activation, serine and tyrosine phosphorylation, nuclear translocation, and the subsequent dephosphorylation and exit from the nucleus (Haspel et al., 1996). Our studies from the immunoprecipitation of cytoplasmic and nuclear extracts confirm this behavior of STAT1α. More interestingly, the cycling of the IFNGRα subunit appears to closely parallel the cycling of tyrosine phosphorylated STAT1α, as determined by immunofluorescence and immunoprecipitation experiments. The IFNGRα receptor subunit is seen to accumulate in the nucleus at the same time as tyrosine phosphorylated STAT1α, and subsequently exit also at the same time as

STAT1α. This time-dependent co-localization of IFNGRα and STAT1α was found to correlate with the antiviral activity of IFNγ, as determined by the immunofluorescence dose-response experiments, suggesting that both the duration and magnitude of receptor activation modulate the nuclear accumulation of IFNGRα and STAT1α and ultimately the biological response.

The nuclear accumulation of IFNGRα is also coincident with the rapid loss of tyrosine (Tyr^{701}) phosphorylated STAT1α from the cytoplasm. The appearance of IFNGRα and STAT1α in the nucleus correlates with low levels of IFNGRα/STAT1α complexes in the cytoplasm. STAT1α is required to bind the IFNGRα subunit following ligand binding for it to be tyrosine phosphorylated on Tyr^{701}, which then activates its nuclear translocation. Thus, this suggests that IFNGRα and activated STAT1α probably remain complexed up to the nuclear import of the two molecules. These observations provide a framework for further studies examining the functional role of the nuclear translocation of IFNGRα, especially its contribution to the nuclear delivery of activated STAT1α molecules.

In addition to establishing the importance of the IFNγR, we have also provided evidence as to the importance of intracellular IFNγ, namely the C-terminal region. We have shown that internalized IFNγ interacts at an intracellular site to regulate the nuclear translocation of STAT1α. This intracellular function of IFNγ is specifically mediated by a C-terminal domain of IFNγ encompassed by residues 95-133, which also contains a NLS that is required for its ability to function intracellularly. Intracellular IFNγ can be recovered as part of a complex with STAT1α in IFNγ activated cells. This complex also contains the nuclear importin-α

analog Npi-1, which has previously been shown to mediate the nuclear import of STAT1α. Further studies showed that the NLS of IFNγ is required for the ability of STAT1α and IFNγ to form this trimeric complex with Npi-1. The formation of the complex IFNγ/Npi-1/STAT1α complex and the subsequent nuclear translocation of STAT1α were all found to be dependent on the presence of the IFNγ NLS. Previous mutational studies on STAT1α failed to identify an NLS motif responsible for the nuclear import of STAT1α (Sekimoto et al., 1997) via Npi-1. Our data strongly support the conclusion that the required NLS for the nuclear localization of STAT1α is provided by IFNγ, and IFNγ acts as a chaperone for the nuclear delivery of STAT1α in the form of a IFNγ:STAT1α:Npi-1 complex.

Earlier studies on the binding of STAT1α to Npi-1 have suggested that STAT1α binds to the C-terminus of Npi-1 (residues 456-538) at a site that cannot be competed for by the basic SV-40 T-NLS (Sekimoto et al., 1997), suggesting that STAT1α binds outside of the "conventional" NLS binding site. However, an alternate interaction site for basic NLSs has been identified with the C-terminal residues 501-510 (Moroianu et al., 1996), which falls within the STAT1α binding region described on Npi-1 (Sekimoto et al., 1997). Interaction of the IFNγ NLS at this site on Npi-1 would provide a mode for binding of an IFNγ/STAT1α complex to Npi-1 in an IFNγ NLS-dependent fashion. Further, amino acid differences within NLSs are known to alter both the specificity and affinity for importin binding sites. Thus, while the SV-40 T-NLSs would qualitatively bind both sites on Npi-1, its affinity for the C-terminal STAT1α site on Npi-1 may be too low to compete with the high specific binding of the IFNγ NLS within the IFNγ/STAT1α complex. This

would explain why in the presence of STAT1α already bound to Npi-1, the SV-40 T-NLS cannot displace STAT1α, as determined in the earlier studies (Sekimoto et al., 1997). Different binding sites for IFNγ NLS and SV-40 T-NLS are also consistent with the ability of SV-40 T-NLS to "compete" in functional assays (Subramaniam et al., 1998), since in functional import assays utilization of Npi-1 by excess SV-40 T-NLS would prevent import of IFNγ. Further studies into the binding of IFNγ to Npi-1 should help provide insight into these phenomenona.

While our data demonstrate that the interaction of IFNγ with Npi-1 regulates the trafficking of STAT1α to the nucleus, it remains to be determined what the exact contribution of intracellular IFNγ is to signaling events that lead to the formation of this complex with STAT1α. Studies from our laboratory and that of others provide strong support for one mechanism by which intracellular IFNγ may regulate early receptor events leading to the activation of the JAK/STAT pathway (Johnson et al., 1998a,b), and the subsequent nuclear translocation of STAT1α. As has been mentioned before, the mouse IFNγ (95-133) and human IFNγ (95-134) peptides, which contain the NLS, are agonists of IFNγ when delivered intracellularly (Johnson et al., 1998a,b). Since it is well known that the ability of IFNγ to induce MHC Class II and an antiviral state is dependent on a functional JAK/STAT pathway (Darnell, 1998), the agonist peptides must be able to activate the JAK/STAT pathway intracellularly to manifest their biological effects. The NLS motif is also required for the agonist properties of the peptide (Szente et al., 1994). Thus, the intracellular interaction of the NLS motif of IFNγ is required for STAT1α nuclear uptake.

As mentioned above STAT1α appears to be deficient in a NLS that can mediate its nuclear import (Sekimoto et al., 1997). Hence we propose that the C-terminal domain of IFNγ, subsequent to its role in recruitment of STAT1α to IFNGRα and priming of STAT1α at IFNGRα for nuclear translocation, via its NLS motif may directly interact with the Ran/importin pathway to mediate the nuclear delivery of STAT1α via a IFNγ/IFNGRα/STAT1α complex. This proposal is consistent with our recent findings that following extracellular binding of IFNγ to the receptor complex IFNGRα is selectively translocated to the nucleus. This nuclear translocation of IFNGRα occurs with the same kinetics as that of STAT1α, and IFNGRα appears to co-localize with STAT1α during this time. IFNGRα and STAT1α can also be recovered as a complex during these processes following IFNγ treatment. We have shown in this study that IFNγ similarly can also be recovered as a complex with STAT1α from the cytoplasm of IFNγ treated cells. These data, thus, argue for the presence of a IFNγ/IFNGRα/STAT1α complex in STAT1α nuclear import.

One major advantage of such ligand-assisted nuclear chaperoning of STAT transcription factors is the high-degree of specificity that is inherent in sequestering a pool of STAT within the ligand-receptor complex that activated it. This would imply that ligand and/or receptor are/is involved in the specificity of STATs at the level of transcription. Further, this would explain why different ligands with different biological functions on a given cell, activate the same STATs in these cells.

As alluded to within the previous paragraph, IFNγ is not the sole protein that utilizes the STAT signaling pathway. It is interesting to speculate as to what confers the specificity of a cellular response of a given ligand, especially in light of the fact that multiple ligands appear to activate the same STAT molecules but turn on different sets of genes. We propose that this chaperone mechanism may not be limited to IFNγ, but may be a means whereby cytokines which utilize the JAK/STAT pathway may exert their effects intracellularly and at the same time confer specificity. A hypothesis of this nature would suggest that putative NLS sequences could be found within the sequences of many ligands that utilize the JAK/STAT pathway for signal transduction. Table 4 shows that this is indeed the case. Interferon γ, PDGF, and IL-1 for example, are all STAT utilizing proteins which all possess putative NLS sequences. In addition, the nuclear translocation of many JAK/STAT extracellular ligands has been observed (Jans, 1994; Lobie et al., 1994; Podlecki et al., 1987; Grenfell et al., 1989, and Clevenger et al., 1991).

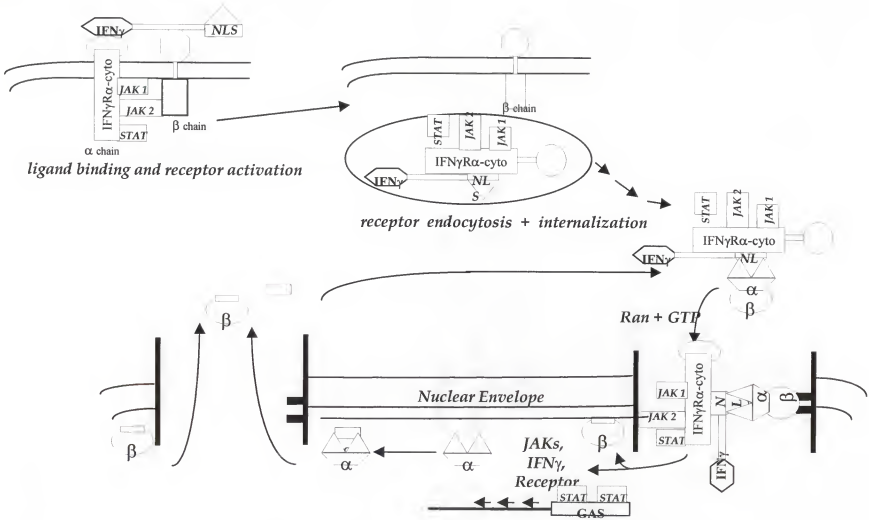
We have proposed a model depicting the direct involvement of IFNγ in the nuclear translocation of STAT 1 that is illustrated within Figure 21. In this model IFNγ first binds to the extracellular domain of the IFNGR via its n-terminus and then undergoes receptor mediated endocytosis. IFNγ then binds to IFNGRα cytoplasmic domain via its c-terminus, The janus kinases are subsequetly activated via phosphorylation and in turn phosphorylate the docking site of STAT 1 on the IFNGRα. STAT1 then binds to the cytoplasmic domain of the IFNGRα. This complex of the IFNGRα, STAT1, and the NLS containing IFNγ is then translocated to the nucleus through the use of the importin pathway.

Table IV. Putative NLS sequences found on some STAT-Utilizing Ligands[a]

Ligand	NLS motif
human PDGF (ligand)	PRESGKKRKRKR
human IFNγ (ligand)	AAKTGRKRKS
human IL-1α (ligand)	KTGKRKRS
human IL-7 (receptor)	KKRIKPVVWPSLPDHKK
human IL-10 (receptor)	RRRKKLPSVLLFKK
human IL-11 (receptor)	KKPFPEDLKSLDLFKKEK
hGM-CSF (receptor)	RNSKRRREIR

[a]Sequences obtained from Johnson et al., 1998

Figure 21 Proposed Nuclear transport of STAT1 model. Data presented provides evidence that supports the nuclear translocation of STAT1 being mediated by the IFNGRα and IFNγ. In this model IFNγ provides the NLS (which binds to importinα and mediates nuclear transport).


IFNγ
NLS
IFNγRα-cyto
JAK 1
JAK 2
STAT
β chain
α chain
ligand binding and receptor activation
β-chain
STAT
JAK 2
JAK 1
IFNγRα-cyto
IFNγ
NL
S
receptor endocytosis + internalization
STAT
JAK 2
JAK 1
IFNγRα-cyto
IFNγ
NL
α
β
Ran + GTP
β
Nuclear Envelope
JAK 1
JAK 2
STAT
IFNγRα-cyto
N
L
α
β
β
α
α
JAKs,
IFNγ,
Receptor
β
IFNγ
STAT
STAT
GAS

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BIOGRAPHICAL SKETCH

Joseph Larkin III was born in Savannah, GA on December 18, 1973 to Mr. and Mrs. Joseph Larkin, Jr. He completed his primary education at the Hodge and Hesse elementary schools located in Savannah, Georgia. Joseph's parents instilled in him at an early age a desire for regular church attendance which continues to be a source of strength to him to this day. From the rigors of Advanced Academic Placement at Jenkins High, Joseph found refuge in playing sports (mostly soccer). Joseph graduated from Jenkins High School in 1992.

Very privileged to be a National Merit Scholar, Joseph was offered an academic scholarship to the University of Florida that he eagerly accepted... primarily for his love of football. Joseph majored in Microbiology, the major of choice for probably half of all premed students. Volunteer work in the hospital and undergraduate research in the laboratory of Dr. Howard M. Johnson finally convinced Joseph to pursue another sort of doctoral degree. After his graduation from the University of Florida in the Spring of 1996 with a Bachelor of Science in Microbiology, Joseph gave up the steel-toed boots of the papermill where he had spent the past five summers, and enrolled in graduate school to continue working in Dr. Johnson's laboratory. After graduation, Joseph plans to accept a postdoctoral position and begin the next phase of his life.

I certify that I have read this study and in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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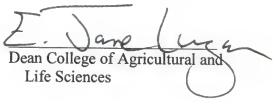

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